IMPROVED REAGENTS FOR RECOMBINOGENIC ENGINEERING AND USES THEREOF

Related Applications

5   This application claims the benefit of U.S. Provisional patent application Serial No. 60/450,474, entitled "Improved Reagents for Recombinogenic Engineering and Uses Thereof", filed February 26, 2003 (pending). The entire content of the above-referenced patent application is hereby incorporated by this reference.

Government Rights

10   This invention was made at least in part with government support under grant no. R21-GM62482 awarded by the National Institutes of Health. The government may have certain rights in this invention.

Background of the Invention

15   A new method for engineering bacterial chromosomes has emerged in recent years that takes advantage of the high proficiency of bacteriophage recombination systems acting on linear DNA substrates (for review, see Court *et al.*, 2002). The λ Red recombination system, consisting of Bet (a ssDNA annealing protein) and Exo (a 5'-3' dsDNA exonuclease) promotes gene replacement of electroporated linear DNA substrates into the *Escherichia coli* K-12 chromosome at a very high efficiency (Murphy, 1998; Murphy *et al.*, 2000). Inactivation of host RecBCD exonuclease activity, either by mutation or production of the anti-RecBCD λ Gam function, is required for efficient Red-promoted recombination with linear dsDNA substrates (Murphy, 1998). Zhang and co-workers (Zhang *et al.*, 1998), using the *E. coli* rac prophage RecET recombination system, recognized that PCR-generated substrates with as little as 40 bp of homology could serve as efficient substrates for gene replacement in *E. coli*. The use of such substrates has also been demonstrated with the λ Red system, with Red and Gam being supplied from either a prophage (Yu *et al.*, 2000), a low-copy number plasmid (Datsenko and Wanner, 2000), or from a Δ*recBCD::Ptac-gam-red* chromosomal substitution (Murphy, 1998; unpublished observations). The high efficiency of Red and RecET-promoted recombination with such short regions of homology has allowed *E. coli* geneticists to perform oligo-directed gene replacements

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that yeast geneticists have performed for years (Baudin *et al.*, 1993; Lorenz *et al.*, 1995; Wach *et al.*, 1994).

Summary of the Invention

5   The present invention features improved methods and systems for promoting recombination in bacteria. In particular, the invention features an improved λ Red recombination system. This improved system is particularly suited for recombination in pathogenic strains of bacteria.

The present invention provides isolated nucleic acid molecules and

10   vectors encoding bacteriophage recombinases, *e.g.*, bacteriophage λ Red and Gam, which are operably linked to a promoter, *e.g.*, Ptac promoter, and the LacI repressor. The bacteriophage recombinases promote homologous recombination between nucleic acid material. Preferably the vectors of the invention further consist of a temperature-sensitive origin of replication that confers low copy number upon the vector.

15   The present invention also features recombinant organisms, *e.g.*, bacteria or pathogenic bacteria, which contain vectors of the present invention and methods of using the recombinant organisms to promote efficient recombination of genetic material. The genetic material undergoing recombination can be endogenous or exogenous, and can be derived from a prokaryote or a eukaryote.

20   The present invention further provides methods of identifying potential drug targets in a recombinant microorganism of the invention, *e.g.*, a pathogenic bacterium, by promoting recombination between a gene of the microorganism and an integrating segment introduced by a test construct. Preferably the integrating segment encodes a selectable marker.

25   The present invention also provides methods of producing vaccines and vaccine antigens.

Other features and advantages of the invention will be apparent from the following detailed description and claims.

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Brief Description of the Drawings

Figure 1. Schematic showing substrates used for λ Red-mediated recombination, and the relative positions of various PCR primers used to generate the substrates and/or verify the structures of chromosomal gene replacements. Chromosomal gene replacements were verified by PCR using primers complementary to sequences upstream (U), downstream (D), N-terminal (N) or C-terminal (C) to the replaced gene, outside the region targeted by PCR (O), within the drug marker (M) and/or within the target gene (T). The absence of the wild type locus (shaded grey) was verified for strains listed in Table 1.

(A.) Plasmids containing marked deletions of target genes were generated as described in Table 2, using primers U, N, C and D as described previously (Murphy, et al., 2000). Plasmid digests (or purified DNA fragments) containing a drug marker flanked by 1-1.5 kb of sequences upstream and downstream of the target gene were electroporated into EHEC and EPEC cells containing Red-producing plasmid pTP223.

(B.) PCR products, containing a drug marker flanked by 40-60 bp of target DNA, were generated by primers designated 3KO and 5KO (see Table 3) and electroporated into EHEC containing pKM201 or pKM208 (or EPEC containing pTP223).

Figure 2. Plasmid-borne *tir* is expressed and translocated at higher levels than chromosomally-encoded *tir*.

(A.) Tir molecules expressed in EPEC are depicted. EHEC Tir sequences are shown in open boxes, EPEC Tir sequences are shown in shaded boxes, and N-terminal HA-epitope tags are shown in black. Tir-PPP is wild type EPEC Tir; chimeric Tir-PHP consists of the N- and C-terminal cytoplasmic domains of EPEC Tir and the extracellular (intimin-binding) domain of EHEC Tir; chimeric Tir-HHH$_{NBS}$ contains the twelve amino acid Nck-binding site of EPEC Tir in the context of an otherwise EHEC Tir. Each of these versions of Tir were encoded on a low copy number plasmid in strain KC26, an EPEC strain which expresses the EHEC versions of *cesT* and *eae* and contains an in-frame deletion of *tir* (Campellone *et al.*, 2002). Alternatively, each of these versions of *tir* were inserted into the chromosome of KC13 at the endogenous Tir locus (see Experimental Procedures).

(B.) HeLa cells were infected with EPEC strains expressing three HA-tagged versions of Tir depicted in (A) either chromosomally or on plasmids. Non-intimately associated bacteria were killed with gentamicin, and the remaining infected HeLa cell monolayers were collected, processed by immunoblot, and probed with anti-HA antiserum to visualize Tir. Blots were

also probed with anti-N-WASP and anti-OmpA antisera to identify the relative cellular and bacterial protein levels, respectively, in each sample. HeLa cell lysates infected with strains harboring *tir* on a plasmid ("P") contain higher protein levels of bacterially-associated (~78kDa) Tir, as well as greater levels of translocated (~90kDa) Tir, than lysates infected with

5 strains harboring *tir* encoded chromosomally ("C").

Figure 3. EPEC expressing chromosomally-encoded Tir-HHH$_{NBS}$ generates pedestals of increased length on mammalian cells.

(A.) HeLa cells were infected with EPEC expressing plasmid-derived Tir molecules and examined microscopically. Monolayers were stained with anti-HA antiserum to visualize

10 translocated Tir (green) and TRITC-phalloidin to visualize F-actin (red). F-actin staining indicated that each version of Tir triggered the formation of pedestals of similar appearance.

(B.) HeLa cells were infected with EPEC expressing chromosome-derived Tir molecules and examined microscopically. Monolayers were processed as in (A). F-actin staining indicated that bacteria expressing chromosomally-derived Tir-HHH$_{NBS}$ generated pedestals of greater

15 lengths than other Tir molecules.

Figure 4. Uncontrolled λ Red expression is mutagenic. A single fresh colony of EHEC (with indicated plasmids) was suspended in 1 ml LB containing 100 µg ml^{-1} ampicillin. The cell suspension was diluted with LB-ampicillin to a final concentration of 5 x 10^4 cells/ml and

20 aliquoted to 24 culture tubes. Overnight cultures (0.3 ml each) of EHEC strain TUV93-0 with control plasmid (circles), pKM201 (squares), pKM208 (triangles) and pKM208 with IPTG added (diamonds) were plated on LB plates containing 100 µg ml^{-1} rifampicin. Plates were incubated overnight at 37 degrees and rifampicin resistant colonies were counted 24 hours later.

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Figure 5. Time course for promotion of hyper-rec phenotype. EHEC strain TUV93-0 containing pKM208 (five cultures, 20 ml each) was grown for electrocompetence as described in Experimental Procedures. At various times prior to collection, IPTG was added to four of the cultures to a final concentration of 1 mM; the fifth culture received no IPTG. The cells

30 were heat shocked for the final 15 minutes, prepared for electroporation and electroporated with DNA (~0.25 µg) containing the *kan* gene flanked by 40 bp of EHEC DNA (resulting in a deletion of O-islands #130 and #131). After suspension in LB, the cells were grown for 90 minutes at 37°C and plated on LB plates containing 20 µg ml^{-1} kanamycin. The number of

kan^R transformants per 10^8 survivor and total number of kan^R transformants are plotted as a function of IPTG concentration. The data points are averages of two experiments (ranges are shown). A random check of 160 colonies showed that 95% re-struck successfully to fresh LB plates containing 20 $\mu g\ ml^{-1}$ kanamycin; 10 of 10 of these colonies were verified by PCR

5 analysis to be true recombinants (data not shown). **Insert**: 0.1 ml of electrocompetent cells, prepared with and without 1 hour IPTG induction, were spread on LB plates containing 100 $\mu g/ml$ rifampicin to determine total number of Rif^R mutants. Dilutions of the cells were titered on LB plates to determine total cell number; experiments done in triplicate (+/- standard error).

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Detailed Description of the Invention

The λ Red recombineering technology has been used extensively in *Escherichia coli* and *Salmonella typhimurium* for easy PCR-mediated generation of deletion mutants, but less so in pathogenic species of *E. coli* such as EHEC and EPEC.

15 The present invention is based, at least in part, on the identification of factors that improve the efficiency of Red recombineering in these pathogenic strains of *E. coli*. The inventors have identified conditions that optimize the use of λ Red for recombineering in EHEC and EPEC. Using plasmids that contain a P_{tac}-*red-gam* operon and a temperature-sensitive origin of replication, multiple mutations (both

20 marked and unmarked) were generated in known virulence genes. In addition, five O157-specific islands (O-islands) of EHEC suspected of containing virulence factors were easily deleted. The inventors have discovered that the both PCR-generated substrates (40 bp of flanking homology) and plasmid-derived substrates (~1 kb of flanking homology) work well, each providing particular advantages. The establishment

25 of the hyper-rec phenotype requires only a 20 minute IPTG induction period of *red* and *gam*. This recombinogenic window is important as constitutive expression of *red* and *gam* induces a 10-fold increase in spontaneous resistance to rifampicin. Other factors such as the orientation of the drug marker in recombination substrates and heat shock effects also play roles in the success of Red-mediated recombination in EHEC and

30 EPEC.

Thus, in the present invention, the λ Red recombineering technology has been optimized for use in pathogenic species of *E. coli*, namely EHEC and EPEC. Exemplifying the utility of this technology, five O-islands of EHEC were easily and

precisely deleted from the chromosome by electroporation with PCR-generated substrates containing drug markers flanked with 40 bp of target DNA. The discoveries described herein are applicable to λ Red recombineering in these and other strains of pathogenic bacteria for faster identification of virulence factors and the speedy

5   generation of bacterial mutants for vaccine development.

Accordingly, the present invention features improved methods and systems for promoting recombination in bacteria. In particular, the invention features an improved λ Red recombination system. This improved system is particularly suited for recombination in pathogenic strains of bacteria. In particular, the invention features

10   isolated nucleic acid molecules and vectors encoding bacteriophage recombinases, *e.g.,* bacteriophage λ Red and Gam, which are operably linked to a promoter, *e.g.,* Ptac promoter, and the LacI repressor. The bacteriophage recombinases promote homologous recombination between nucleic acid material. Preferably the vectors of the invention further consist of a temperature-sensitive origin of replication that confers low

15   copy number upon the vector.

A featured vector of the invention is pKM208. pKM208 expresses Red and Gam and possesses a low copy number replicon which is temperature sensitive. Red and Gam are expressed from the *Ptac* promoter, a promoter capable of directing high levels of expression of the red and gam genes. High levels of Red and Gam result

20   in efficient gene replacement, preferably, when this plasmid is used in pathogenic bacteria (*e.g.,* pathogenic *E. coli* species). The data exemplified herein shows that pKM208 promotes both long and short homology gene replacement in enterohemorrhagic *E. coli* 0157:H7 (EHEC, a pathogen for come concern in the cattle industry).

25   The present invention also features recombinant organisms, *e.g.,* bacteria or pathogenic bacteria, which contain vectors of the present invention and methods of using the recombinant organisms to promote efficient recombination of genetic material.

The recombination systems of the invention are particularly useful in drug target validation. For example, the systems can be used to make knockouts of

30   suspected virulence and/or essential genes, thereby identifying potential drug targets. The recombination systems are also useful in vaccine development, in particular, in the development of vaccines against *E. coli* pathogens. The systems are also useful for *in*

vivo cloning. In particular, the systems can be used for the cloning of vaccine antigens against EHEC.

In order that the present invention may be more readily understood,
5   certain terms are first defined herein.

The term "nucleic acid molecule" refers to a polymer of nucleotides, preferably, deoxyribonucleotides or ribonucleotides or analogs of said nucleotides, for example, analogs having modified binding properties and/or metabolic properties. The
10   term "nucleic acid molecule" is intended to include DNA molecules (*e.g.*, cDNA or genomic DNA) and RNA molecules (*e.g.*, mRNA) and analogs of the DNA or RNA generated using nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA.

An "isolated" nucleic acid molecule is one which is separated from other
15   nucleic acid molecules which are present in the natural source of the nucleic acid. For example, with regards to genomic DNA, the term "isolated" includes nucleic acid molecules which are separated from the chromosome with which the genomic DNA is naturally associated. Preferably, an "isolated" nucleic acid is free of sequences which naturally flank the nucleic acid (*i.e.*, sequences located at the 5' and 3' ends of the
20   nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated EPK-55053 nucleic acid molecule can contain less than about 5 kb, 4kb, 3kb, 2kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. Moreover, an "isolated" nucleic acid
25   molecule, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized.

The term "gene", as used herein, includes a nucleic acid molecule (*e.g.*, a DNA molecule or segment thereof), for example, a protein- or RNA-encoding nucleic
30   acid molecule, that in an organism, is separated from another gene or other genes, by intergenic DNA (*i.e.*, intervening or spacer DNA which naturally flanks the gene and/or separates genes in the chromosomal DNA of the organism). A gene may direct synthesis of an enzyme or other protein molecule (*e.g.*, may comprise coding sequences,

for example, a contiguous open reading frame (ORF) which encodes a protein) or may itself be functional in the organism, *e.g.*, may function as a recognition sequence for proteins in the organism. A gene in an organism, may be clustered in an operon, as defined herein, said operon being separated from other genes and/or operons by the

5   intergenic DNA. Individual genes contained within an operon may overlap without intergenic DNA between said individual genes.

An "isolated gene", as used herein, includes a gene which is essentially free of sequences which naturally flank the gene in the chromosomal DNA of the organism from which the gene is derived (*i.e.*, is free of adjacent coding sequences

10   which encode a second or distinct protein or RNA molecule, adjacent structural sequences or the like) and optionally includes 5' and 3' regulatory sequences, for example promoter sequences and/or terminator sequences. Preferably, an isolated gene contains less than about 10 kb, 5 kb, 2 kb, 1 kb, 0.5 kb, 0.2 kb, 0.1 kb, 50 bp, 25 bp or 10 bp of nucleotide sequences which naturally flank the gene in the chromosomal DNA

15   of the organism from which the gene is derived.

Genes can be obtained from a variety of sources, including cloning from a source of interest or synthesizing from known or predicted sequence information, and may include sequences designed to have desired parameters.

A DNA segment is "operably linked" when placed into a functional

20   relationship with another DNA segment. For example, DNA encoding a signal peptide is operably linked to DNA encoding a protein or polypeptide if, when expressed, the sequences encode the signal peptide in frame with the protein or polypeptide. Likewise, a promoter or enhancer is operably linked to DNA encoding a protein or polypeptide if expression of the protein or polypeptide is promoted or enhanced. In one embodiment,

25   DNA sequences that are operably linked are contiguous (*e.g.*, in the case of a signal sequences). Alternatively, DNA sequences that are operably linked can be non-contiguous (*e.g.*, in the case of enhancers).

"Promoter" refers to a region of DNA involved in binding a polymerase (*e.g.*, a RNA polymerase) to initiate transcription. An "inducible promoter" refers to a

30   promoter that directs expression of a gene where the level of expression is alterable by environmental or developmental factors such as, for example, temperature, pH, transcription factors, activators, repressors and chemicals.

An "expression cassette" is a nucleic acid construct, generated recombinantly or synthetically, with nucleic acid elements that are capable of effecting expression of a structural gene in hosts compatible with such sequences. Expression cassettes include at least promoters and optionally, transcription termination signals. Typically, the recombinant expression cassette includes a nucleic acid to be transcribed (*e.g.*, a nucleic acid encoding a desired polypeptide), and a promoter. Additional factors necessary or helpful in effecting expression may also be included. For example, transcription termination signals, enhancers, and other nucleic acid sequences that influence gene expression, can also be included in an expression cassette.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Alternatively, a vector can be linear.

The term "recombinant vector" includes a vector that has been altered, modified or engineered such that it contains greater, fewer or different nucleic acid sequences than those included in a naturally-occurring vector.

As used herein, "origin of replication sequences" refer to sequences which, when present in a vector, initiate replication. Origin of replication sequences "which confer low copy number on a vector" refer to sequences which, when present in a vector, direct replication of the vector such that it is maintained in the host cell/organism at a low copy number.

As used herein, a "temperature sensitive" origin of replication refers to a replication origin which is controlled by temperature, *e.g.,* is rendered nonfunctional at a nonpermissive temperature and/or is functional at a permissive temperature.

As used here, the term "exogenous" refers to genetic material (*e.g,* nucleic acid material") that originates from a source foreign to the particular host organism, or, if from the same source, is modified from its original form. The term "endogenous" refers to genetic material (*e.g,* nucleic acid material) that originates from the host organism, *i.e.*, that is naturally-occurring.

As used herein, "recombination" refers to a process by which nucleic acid material *e.g.*, DNA, is exchanged between two nucleic acid molecules, for example, in a microorganism. As used herein, "homologous recombination" refers to a process by

which nucleic acid material *e.g.*, DNA, is exchanged between two nucleic acid molecules through regions or segments of sequence homology, or preferably, sequence identity (*e.g.*, a high degree of sequence identity). In exemplary embodiments, the nucleic acid material, *e.g.*, DNA, is located on a chromosome or an episome of the

5   microorganism. In another exemplary embodiments, the nucleic acid material, *e.g.*, DNA, is located extrachromasomally, for example, on a plasmid. Recombination can occur between linear and/or circular DNA molecules.

As used herein, "recombinase" refers to an enzyme, enzymatic activity or enzymatic function that catalyzes recombination. Preferred recombinases of the present

10   invention catalyze homologous recombination. Particularly preferred recombinases of the present invention are bacteriophage recombinases, for example, the bacteriophage λ Red recombinase (encoded by *exo* and *bet* nucleotide sequences).

As used herein, "anti-recombinase" refers to inhibitor of a recombinase activity endogenous to the host organism. Preferred anti-recombinases of the invention

15   are bacteriophage anti-recombinases (*e.g.*, the bacteriophage anti-recombinase encoded by *gam*) which inhibits RecBCD in the host organism.

As used herein, the phrase "recombination segments" refers to segments of nucleic acid material (*e.g.*, DNA) that are sufficiently homologous or identical to target nucleic sequences, for example, sequences present in or within the vicinity of a

20   gene present in a microorganism (*e.g.*, within < 1kB of the gene), such that the segment directs recombination at the target nucleic acid sequence. The recombination segments are routinely separated by nucleic acid material (*e.g.*, DNA) that is to be integrated at the target site. Recombination segments routinely are recognized by the recombinase enzymes described herein. Recombination segments are preferably between 40-60 base

25   pairs (bp) in length, *i.e.*, are homologous or identical to a region of target DNA of between 20-80, 30-70, or 40-60 bp in length (*e.g.*, 50 bp in length).

As used herein, an "integrating segment" refers to a nucleotide sequence that is to be integrated at a target recombination site in nucleic acid of a microorganism. The integrating segment can be random, *e.g.*, spacer nucleic acid or, preferably, nucleic

30   acid encoding for a selectable marker, *e.g.*, ampicillin or kanamycin. An integrating segment useful in the present invention is flanked by recombination segments. As used herein, nucleic acid "flanked" by recombination segments indicates that the

recombination segments are located 5' and 3' to the nucleic acid (*e.g.*, left and right arms).

As used herein, the term "substrate" refers to nucleic acid material, *e.g.*, DNA, that includes at least recombination segments, as defined herein. Preferred

5 substrates further include an integrating segment flanked by said recombination segments. A particularly preferred substrate is a linear double-stranded ("ds") or duplex DNA molecule. An exemplary substrate is less than 2.5 kb in length.

As used herein, the phrase "recombination proficient" refers to a microorganism in which homology-dependent or homologous recombination can occur.

10 Notably, homologous recombination occurs at some level (*e.g.*, at a baseline level) in naturally occurring microorganisms. Preferred "recombination proficient" microorganisms of the instant invention have been engineered such that homologous recombination occurs at a level greater than that observed in corresponding naturally occurring microorganisms. Recombinant microorganism which have been so

15 engineered are also referred to herein as "hyper-recombination proficient" microorganisms.

As used herein, the term "derived from", when referring to a prokaryote or a eukaryote, includes a nucleic acid or gene which is naturally found in a prokaryotic organism or a eukaryotic organism. Preferably, the nucleic acid or gene is derived from

20 a microorganism, *e.g.*, a bacteria, *e.g.*, *Escherichia coli*. The term "bacterially-derived" or "derived-from", for example bacteria, includes a gene which is naturally found in bacteria or a gene product (*e.g.*, an enzyme) which is encoded by a bacterial gene.

The term "operon" includes a coordinated unit of gene expression that contains a promoter and possibly a regulatory element associated with one or more,

25 preferably at least two, structural genes (*e.g.*, genes encoding enzymes). Expression of the structural genes can be coordinately regulated, for example, by regulatory proteins binding to the regulatory element or by anti-termination of transcription. The structural genes can be transcribed to give a single mRNA that encodes all of the structural proteins. Due to the coordinated regulation of genes included in an operon, a single

30 promoter and/or regulatory element can control expression of each gene product encoded by the operon.

I.   Bacteriophage recombinase functions and recombination-promoting vectors

The present invention features recombination-promoting vectors for use in a variety of recombination methods. The vectors include sequences encoding

5   bacteriophage recombinases, *e.g.*, bacteriophage λ Red and Gam, which are operably linked to a promoter, *e.g.*, Ptac promoter, and the LacI repressor. The bacteriophage recombinases promote homologous recombination between nucleic acid material. Preferably the vectors of the invention further consist of a temperature-sensitive origin of replication that confers low copy number upon the vector.

10   The nucleic acid molecules and vectors of the present invention provide a bacteriophage recombinase and/or antirecombinase function. In preferred embodiments, the bacteriophage recombinase function is a bacteriophage λ Red recombinase function and the antirecombinase function is a λ Red antirecombinase function.

The nucleic acid molecules and vectors of the invention preferably

15   include nucleotide sequences encoding the bacteriophage λ gene product Exo or a functional equivalent thereof, Bet or a functional equivalent thereof, and Gam or a functional equivalent thereof. The *exo, bet* and *gam* genes (and their corresponding gene products) are well known to those skilled in the art and the coding sequences of those genes have the following GenBank accession numbers: (1) *exo* gene:

20   ACCESSION NC_001416, REGION complement (31348..32028), VERSION NC_001416.1 GI:9626243, Gene ID: 2703522, (SEQ ID NO: 35); (2) *bet* gene: ACCESSION NC_001416, REGION complement (32025..32810), VERSION NC_001416.1 GI:9626243, Gene ID: 2703535, (SEQ ID NO: 36); (3) *gam* gene: ACCESSION NC_001416, REGION complement (32816..33232), VERSION

25   NC_001416.1 GI:9626243, Gene ID: 2703509, (SEQ ID NO: 37). The corresponding amino acid sequences have the following GenBank accession numbers: Exo: Accession no: NP_040616 - GI:9626280; Bet - Accession no: NP_040617 - GI:9626281; Gam - Accession no: NP_040618 - GI:9626282.

30   As used herein the term "functional equivalent" refers to gene product which can provide a similar enzymatic activity to the gene products having the GenBank accession numbers given above. A functional equivalent of Exo, Bet or Gam has a similar enzymatic activity in the sense that it catalyses the same reaction with substantially the same specificity as Exo, Bet or Gam and catalyses that reaction at a rate

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of at least 60%, at least 70%, preferably at least 80%, generally at least 90%, for example at least 95%, typically at least 99% and most preferably at a rate substantially the same as that of Exo, Bet or Gam when measured under the same conditions.

Functional equivalents of Exo, Bet or Gam may be obtained by any method known to those skilled in the art. For example, they can be generated by nucleotide substitutions of the wild type sequence of *exo, bet* or *gam*, for example from 1, 2 or 3 to 10, 25, 50 or 100 substitutions. Wild type sequences may alternatively or additionally be modified by one or more insertions and/or deletions and/or by an extension at either or both ends to give functional equivalents. The modified polynucleotide typically encodes a gene product which has activity as defined above.

Degenerate substitutions may be made and/or substitutions may be made which would result in a conservative amino acid substitution when the modified sequence is translated. Typically a functional equivalent will share at least 70% identity, at least 80% identity, at least 90% identity, at least 95% identity, or at least 99% identity with the wild type Exo, Bet or Gam sequence over at least 20, preferably at least 30, for instance at least 40, at least 60, or more preferably at least 100 contiguous amino acids or most preferably over the full length of the wild type Exo, Bet or Gam sequence.

The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch (*J. Mol. Biol.* (48):444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package (available at on line through the Genetics Computer Group), using either a Blosum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available online through the Genetics Computer Group), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. A preferred, non-limiting example of parameters to be used in conjunction with the GAP program include a Blosum 62 scoring matrix with a gap penalty of 12, a gap extend penalty of 4, and a frameshift gap penalty of 5.

In another embodiment, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of Meyers, E. and Miller, W.

(*Comput. Appl. Biosci.* 4:11-17 (1988)) which has been incorporated into the ALIGN program (version 2.0 or version 2.0U), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

The nucleic acid and polypeptide sequences of the present invention can

5 further be used as a "query sequence" to perform a search against public databases to, for example, identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul *et al.* (1990) *J. Mol. Biol.* 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences

10 homologous to *exo*, *bet* or *gam* nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 100, wordlength = 3, and a Blosum62 matrix to obtain amino acid sequences homologous to Exo, Bet or Gam polypeptide molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (1997) *Nucleic*

15 *Acids Res.* 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. See the website for the National Center for Biotechnology Information.

A functional equivalent may also be capable of hybridizing under stringent hybridization conditions to a complement of the wild type *exo, bet* or *gam*

20 sequence. Such stringent conditions are known to those skilled in the art and can be found in *Current Protocols in Molecular Biology*, Ausubel *et al.*, eds., John Wiley & Sons, Inc. (1995), sections 2, 4, and 6. Additional stringent conditions can be found in *Molecular Cloning: A Laboratory Manual*, Sambrook *et al.*, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989), chapters 7, 9, and 11. A preferred, non-limiting

25 example of stringent hybridization conditions includes hybridization in 4X sodium chloride/sodium citrate (SSC), at about 65-70°C (or alternatively hybridization in 4X SSC plus 50% formamide at about 42-50°C) followed by one or more washes in 1X SSC, at about 65-70°C. A preferred, non-limiting example of highly stringent hybridization conditions includes hybridization in 1X SSC, at about 65-70°C (or

30 alternatively hybridization in 1X SSC plus 50% formamide at about 42-50°C) followed by one or more washes in 0.3X SSC, at about 65-70°C. A preferred, non-limiting example of reduced stringency hybridization conditions includes hybridization in 4X SSC, at about 50-60°C (or alternatively hybridization in 6X SSC plus 50% formamide at

about 40-45°C) followed by one or more washes in 2X SSC, at about 50-60°C. Ranges intermediate to the above-recited values, e.g., at 65-70°C or at 42-50°C are also intended to be encompassed by the present invention. SSPE (1xSSPE is 0.15M NaCl, 10mM NaH_2PO_4, and 1.25mM EDTA, pH 7.4) can be substituted for SSC (1X SSC is 0.15M

5 NaCl and 15mM sodium citrate) in the hybridization and wash buffers; washes are performed for 15 minutes each after hybridization is complete. The hybridization temperature for hybrids anticipated to be less than 50 base pairs in length should be 5-10°C less than the melting temperature (T_m) of the hybrid, where T_m is determined according to the following equations. For hybrids less than 18 base pairs in length,

10 $T_m(°C) = 2(\text{\# of A + T bases}) + 4(\text{\# of G + C bases})$. For hybrids between 18 and 49 base pairs in length, $T_m(°C) = 81.5 + 16.6(\log_{10}[Na^+]) + 0.41(\%G+C) - (600/N)$, where N is the number of bases in the hybrid, and $[Na^+]$ is the concentration of sodium ions in the hybridization buffer ($[Na^+]$ for 1X SSC = 0.165 M). It will also be recognized by the skilled practitioner that additional reagents may be added to hybridization and/or wash

15 buffers to decrease non-specific hybridization of nucleic acid molecules to membranes, for example, nitrocellulose or nylon membranes, including but not limited to blocking agents (e.g., BSA or salmon or herring sperm carrier DNA), detergents (e.g., SDS), chelating agents (e.g., EDTA), Ficoll, PVP and the like. When using nylon membranes, in particular, an additional preferred, non-limiting example of stringent hybridization

20 conditions is hybridization in 0.25-0.5M NaH_2PO_4, 7% SDS at about 65°C, followed by one or more washes at 0.02M NaH_2PO_4, 1% SDS at 65°C (see e.g., Church and Gilbert (1984) Proc. Natl. Acad. Sci. USA 81:1991-1995), or alternatively 0.2X SSC, 1% SDS.

A sequence which can hybridize to the complement of its corresponding sequence can typically hybridize to that coding sequence at a level significantly above

25 background. The signal level generated by the interaction between a functional equivalent and the complement of its corresponding sequence is typically at least 10 fold, preferably at least 100 fold, as intense as interactions between other sequences and the Exo, Bet or Gam coding sequences.

In preferred embodiments the promoters are regulated, because the exo,

30 bet and gam genes or functional derivatives of any thereof can then be expressed only when homologous recombination is required. This may help to reduce any unwanted recombination events mediated by the exo, bet and gam polypeptides or polypeptides

similar thereto. In preferred embodiments, a promoter such as Ptac in combination with LacI repressor may be used.

It is also within the scope of the invention that a prophage can be used to express *exo, bet* and *gam* (or genes encoding functional derivatives of any thereof). If a prophage is used to express *exo, bet* and *gam* (or functional derivatives), it may be convenient to engineer controlled expression using the cI-repressor. Use of this system allows expression of *exo, bet, and gam* to be controlled by temperature. Growth of cells containing the prophage at 32°C results in no expression of *exo, bet* and *gam*, whereas growth of cells containing the prophage at 42°C results in expression of *exo, bet* and *gam*. Use of such a system may require cells containing the prophage to be grown to be at the permissive temperature for a short time, for example from 2 to 30 minutes, for example from 5 to 15 minutes, before transfer of the construct into the cells.

Vectors which include sequences encoding other bacteriophage recombinases (*e.g,* Rac prophage recombinases encoded by the RecE and RecT genes) are also contemplated within the scope of the instant invention.

II. Substrates

In a preferred embodiment, the substrates for use in the recombination methods of the invention comprise an integrating segment flanked by recombination segments, wherein the recombination segments are homologous to the target bacterial gene or surrounding sequences

A.   Recombination segments

Recombination segments typically flank an integrating segment when in a substrate of the invention and will flank a target nucleotide sequence upon recombination. Such sequences have to be sufficiently similar to the corresponding sequence in the bacterium and of sufficient length for homologous recombination to occur between the substrate and the bacterial chromosome. Typically, recombination segments will be sufficiently dissimilar from each other such that recombination of the target sequence occurs in a selected orientation.

Recombination segments do not need to be of the same length. In general, recombination segments may be, independently, at least 10bp in length, at least 20bp, at least 50 bp in length, at least 60bp in length, at least 75bp in length or at least 100bp in length. Typically, recombination segments will be, independently, up to 200bp

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in length, up to 300bp in length, up to 500bp in length, up to 750bp in length, up to 1 kb in length or up to 2kb in length.

Typically, recombination segments for use in the invention will share 100% identity over their entire length with the target sequence to which they correspond

5 (*e.g.*, the corresponding on the bacterial chromosome). However, recombination segments suitable for use in the invention may share, independently, at least 60% identity, at least 70% identity, at least 80% identity, at least 90% identity, at least 95% identity, or at least 99% identity with the corresponding bacterial sequence over a contiguous stretch of nucleotides representing at least 50% of its length, at least 60% of

10 its length, at least 70% of its length, at least 80% of its length, at least 90% of its length, at least 95% of its length or at least 99% its length. Any combination of the above mentioned percentage identity and percentage length may be used to define a recombination segment suitable for use in the invention, with greater % identity to the corresponding sequence on the bacterial chromosome over a greater percentage of the

15 length of the sequence being preferred.

A suitable recombination segments may also be capable of hybridizing under stringent conditions to the complement of the sequence to which it corresponds on the bacterial chromosome. Such stringent conditions are known to those skilled in the art and can be found in *Current Protocols in Molecular Biology*, Ausubel *et al.*, eds., John

20 Wiley & Sons, Inc. (1995), sections 2, 4, and 6. Additional stringent conditions can be found in *Molecular Cloning: A Laboratory Manual*, Sambrook *et al.*, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989), chapters 7, 9, and 11. A preferred, non-limiting example of stringent hybridization conditions includes hybridization in 4X sodium chloride/sodium citrate (SSC), at about 65-70°C (or alternatively hybridization

25 in 4X SSC plus 50% formamide at about 42-50°C) followed by one or more washes in 1X SSC, at about 65-70°C. Other preferred, nonlimiting examples of stringent hybridization conditions are as described herein.

The λ Red-promoted recombination methods of the invention can be carried out using substrates comprising short recombining segments, e.g., individually,

30 about 40-60 bp. A substrate of the invention comprising short recombination segments can be a PCR-generated substrate. PCR methodologies to produce such substrates are commonly known in the art, and would be apparent to the skilled artisan. PCR-generated substrates offer a simple mechanism for generating gene knockouts. The

λ Red-promoted recombination methods of the invention can be carried out using substrates comprising long recombining segments, e.g., individually, up to about 1 kb or 2kb or more. A substrate of the invention comprising long recombination segments can be a vector or plasmid. Long homology-containing vector, e.g., plasmid, substrates

5 provide advantages, for example, when multiple mutant alleles of a target gene need to be crossed into the chromosome. When multiple mutant alleles of the target gene will be crossed into the chromosome, it is desirable to have the substrate previously cloned, in order to not induce PCR errors into the allele prior to transfer to the chromosome. A dedicated plasmid containing sequenced regions upstream and downstream regions of

10 the target gene is required. Typically, long homology-containing substrates promote higher frequencies of gene replacement relative to short homology substrates. Typically, long homology-containing substrates offer higher success rates for Red-promoted gene replacement in pathogenic hosts that are not as electrocompetent as *E. coli* K-12.

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B.   Integrating segment

The integrating segment may be any sequence with which it is desired to replace the target sequence. The integrating segment does not need to be the same length as the target sequence and can be shorter or longer than the target sequence. In

20 one embodiment, the integrating segment and target sequence are similar in length, for example, the integrating segment may be 50%, 60%, 70%, 80%, 90%, 95% or substantially the same length of the target sequence or vice versa. It will be convenient for the integrating segment to comprise a coding sequence which codes for a polypeptide which is readily detectable (*i.e.*, a marker polypeptide), so that bacteria in

25 which homologous recombination has occurred can be easily identified. If the integrating segment comprises a coding sequence, it will also typically comprise a promoter operably linked to that coding sequence. The promoter should be selected so that expression of the coding sequence will be driven in the bacterium into which the construct is transferred.

30   Exemplary marker polypeptides include drug markers, for example, polypeptides that confer resistance to kanamycin, ampicillin or tetracycline. Alternatively, the marker is a reporter polypeptide. Such a polypeptide may be, for

example, a fluorescent or a colorimetric polypeptide. Such polypeptides are easy to detect using techniques will known to those skilled in the art.

If the marker confers antibiotic resistance on the bacterium, then candidate bacteria can be grown in the presence of the antibiotic. Only bacteria which

5   have successfully incorporated the integrating segment will be able to grow in the presence of the antibiotic. Alternatively, if the marker is a gene encoding a fluorescent polypeptide, for example green fluorescent protein, fluorescence may be used to indicate the presence of the integrating segment.

Substrates for use in the invention can be prepared using, for example,

10   recombinant DNA technology well known to those skilled in the art. Typically, it will be convenient to prepare Substrates using polymerase chain reaction (PCR). Primers may be designed which comprise sequences corresponding to the recombination segments.

The primers described above may be used in PCR on a template which

15   comprises the integrating segment to generate a linear substrate suitable for use in the invention. PCR can be carried out on, for example, a plasmid which contains the integrating segment and the linear PCR product obtained can then be purified from the PCR - reaction mixture. It may, however, be more convenient to isolate the integrating segment as a linear fragment and to carry out PCR on that fragment. Use of this latter

20   technique is advantageous over a technique which carries out PCR directly on a plasmid, because no purification of the construct is required after PCR. In order to ensure that the plasmid does not interfere with the subsequent transfer and selection (if selection is used), the resulting PCR reaction has to undergo a rigorous purification scheme involving: (1) gel purification; (2) digestion with a restriction endonuclease; and (3) a

25   further round of gel purification. Such steps are not required if PCR is carried out on a linear template.

The use of single stranded DNA (ssDNA) substrates and/or vectors is also within the scope of the invention.

30

III. Recombination

The substrate can be introduced into a recombination-proficient bacteria by any suitable method. Suitable methods are will known to those skilled in the art, for

example electroporation or thermal shock. Homologous recombination between the substrate and a target sequence in the bacteria leads to replacement of the target sequence with the integrating segment of the substrate.

A target sequence may comprise all or part of a bacterial gene, for example, the target sequence may comprise all or part of a control sequence, such as a promoter, or may comprise or all or part of a coding sequence. The target sequence may be of any suitable size, for example from about 1 bp to about 50 kb in length. Typically, however, the minimum size of a target sequence will be at least 10 bp, at least 25 bp, at least 50 bp or more, preferably at least 100 bp or typically at least 500 bp in length. In general the maximum length of a target sequence will be up to 30 kb, up to 15 kb, up to 5 kb or up to 2 kb. The length of the target sequence may preferably be up to 1 kb and typically up to 800 bp. Any combination of the above mentioned lower and upper lengths may be used to defined a target sequence of the invention. Recombination segments are preferably within the vicinity of a target gene (*e.g.*, within at least about < 1 kb, 2 kb, 5 kb, 10 kb, 20 kb or more of the target gene). Notably, however, the λ Red systems of the invention are capable of recombining nucleic acid segments as large as 10 kb, 20 kb, 30kb, 40kb, 50 kb or more.

IV.   Host organisms

The invention may be carried out using any bacteria. For example, the bacteria may be a Gram-positive bacteria (*i.e.*, a bacteria which retains basic dye, for example, crystal violet, due to the presence of a Gram-positive wall surrounding the microorganism) or a Gram-negative bacteria (*i.e.*, excludes basic dye). Preferred bacteria are pathogenic bacteria. The bacteria may be pathogenic for a human or an animal or for a plant.

The bacteria may be for example, from the genus *Escherichia*. Preferred pathogenic bacteria are from the *E. coli* species. Enterohemorrhagic *E. coli* O157:H7 (EHEC) and enteropathogenic *E. coli* (EPEC) are members of the attaching and effacing (AE) family of enteric pathogens (reviewed by Nataro and Kaper, 1998 and Vallance *et al.*, 2002). These pathogens bind tightly to the intestinal epithelium and cause localized effacement of micovilli, followed by alteration of the cytoskeleton beneath sites of bacterial attachment. The result of this process is the formation of a filamentous structure known as an actin pedestal (Frankel *et al.,* 1998). The presence of these

pedestals correlate with the ability of EHEC and EPEC to cause disease in mammals (Donnenberg *et al.*, 1993; Tzipori *et al.*, 1995).

Genetic loci responsible for the generation of actin pedestals are located within a pathogenicity island known as the locus of enterocyte effacement, or LEE

5   (McDaniel and Kaper, 1997; Perna *et al.*, 1998). Two genes within this locus that are critical for actin pedestal formation in both EHEC and EPEC are *eae* and *tir*. The product of the *eae* gene, intimin, is an outer membrane protein that is necessary for attachment to the target cell (Jerse *et al.*, 1990). The receptor for intimin, Tir (translocated intimin receptor), is a bacterial protein that is translocated into the host cell

10   membrane by the LEE-encoded type III secretion system (Kenny *et al.*, 1997; Deibel *et al.*, 1998). Following translocation, the interaction of the extracellular central domain of Tir with intimin displayed on the bacterial surface triggers the signals required for actin pedestal formation (Rosenshine *et al.*, 1996; deGrado *et al.*, 1999; Hartland *et al.*, 1999; Kenny, 1999; Liu *et al.*, 1999). Mutants in *eae* or *tir* are unable to establish intimate

15   contact with the host cell, do not form AE lesions, and are diminished for virulence in animal models (Jerse *et al.*, 1990; Yu and Kaper, 1992; Rosenshine, 1996; Liu *et al.*, 2002). Deletions in these genes have previously been reported using classical plasmid-integrant and resolution technology (Donnenberg and Kaper, 1991; DeVinney *et al.*, 1999; McKee *et al.*, 1995), or by low frequency linear DNA transformation of thiolate-

20   containing derivatives (Donnenberg *et al.*, 1993).

The bacteria may also be for example, from the genera *Salmonella, Vibrio, Haemophilus, Neisseria, Yersinia, Bordetella, Brucella, Shigella, Klebsiella, Enterobacter, Serracia, Proteus, Vibrio, Aeromonas, Pseudomonas, Acinetobacter, Moraxella, Flavobacterium, Actinobacillus, Staphylococcus, Streptococcus,*

25   *Mycobacterium, Listeria, Clostridium, Pasteurella, Helicobacter, Campylobacter, Lawsonia, Mycoplasma, Bacillus, Agrobacterium, Rhizobium, Erwinia* or *Xanthomonas*.

Examples of some of the above mentioned genera are *Escherichia coli* - a cause of diarrhea in humans; *Salmonella typhimurium* - the cause of salmonellosis in several animal species; *Salmonella typhi* - the cause of human typhoid fever; *Sal nonella*

30   *enteritidis* - a cause of food poisoning in humans; *Salnzonella choleraesuis* - a cause of salmonellosis in pigs; *Salmonella dublin* - a cause of both a systemic and diarrhoeal disease in cattle, especially of new-born calves; *Haemophilus influenzue* - a cause of meningitis; *Neisseria gonorrhoeae* - a cause of gonorrhoea; *Yersinia enterocolitica* - the

cause of a spectrum of diseases in humans ranging from gastroenteritis to fatal septicemic disease; *Bordetella pertussis* - the cause of whooping cough; *Brucelia abortus* - a cause of abortion and infertility in cattle and a condition known as undulant fever in humans; *Vibrio cholerae* - a cause of cholera; *Clostridium tetani* - a cause of

5   tetanus; and *Bacillus anthracis* - a cause of anthrax.

V.   Uses

The invention provides a general method for promoting efficient

10   recombination of genetic material in a microorganism, *e.g.,* a bacteria, preferably a pathogenic bacteria. The methods provided in the invention can be used to replace a specific genomic sequence in a bacterium with any other desired sequence present in a substrate, as defined herein. The methods of the invention can also be used to replace a specific sequence present on an episome in a bacterium with any other desired sequence

15   present on a substrate. When the specific sequence to be replaced is present on an episome in a bacterium, that sequence can be derived, *e.g.,* from a prokaryote or a eukaryote.

Several rounds of recombination of genetic material according to the invention may be carried out sequentially, each time using a different substrate.

20   Alternatively, more than one, for example, two, three, four , five or more, substrates for use in the invention can be transferred simultaneously into a bacterium. Thus, a bacterium produced using methods of the invention may comprise mutations in more than one, for example two, three, four, five or more, genes.

After successful recombination, it may be necessary to eliminate the

25   integrating segment from the genetically modified bacterium. This may be required if the genetically modified bacterium is to be used in or as a vaccine. For example, the use of antibiotic resistance genes in live attenuated bacteria is not generally permitted by regulatory authorities.

A construct of the invention may therefore also comprise sequences

30   which may be used to eliminate the integrating segment. Such sequences will typically flank the integrating segment and will generally be positioned between the first recombination segment and the integrating segment and between the second recombination segment and the integrating segment.

The present invention further provides methods for determining whether a bacterial gene is a potential drug target. For example, the method allows null mutants to be created, and thus provides an important tool in the analysis of genes for which no function is known. If a particular sequence, for example an open reading frame or a part

5   thereof or a region 5' to an open reading frame (for example a promoter) or part thereof, cannot be replaced, that may indicate that the sequence is essential for viability in the bacterium in which the sequence occurs, i.e. that the sequence represents all or part of an essential gene. An essential gene is a gene which, when missing (*e.g.,* because of a chromosomal deletion) or mutated to render it non-functional, results in a lethal

10   phenotype. That is, a gene without which a bacteria cannot survive. Essential sequences are targets for the development of new antibiotics. For example, bacterial genes identified as new drug targets by the methods of the present invention are used in screening assays for new antimicrobial substances.

The use of bioinformatics may allow the rapid isolation of further

15   essential genes, i.e. corresponding genes from other bacterial species. A gene identified from a particular species by using the methods of the invention may be used to search databases containing sequence information from other species, in order to identify orthologous genes from those species. Genes so identified can then be tested for whether they are essential by using the genetic recombination methods of the invention.

20   For example, if an E. coli gene is identified as essential using a method as described above, this may allow the identification of a putative orthologue from Salmonella. That Salmonella gene may be tested by using the genetic recombination methods of the present invention. Further orthologues may be identified in more distantly related organisms.

25   Suitable bioinformatics programs are well known to those skilled in the art. For example, the Basic Local Alignment Search Tool (BLAST) program (Altschul et al., 1990, J. Mol. Biol. 215, 403-410 and Altschul et al., 1997, Nucl. Acids Res. 25, 30 3389-3402.) may be used. Suitable databases for searching are for example, EMBL, GENBANK, TIGR, EBI, SWISS-PROT and trEMBL.

30

The methods and systems of the invention are also useful in gene repair and replacement methodologies. The methodologies of the invention are also useful in in functional genomics strategies designed to analyze the genomes of microorganisms,

i.e., to determine the function of unknown genes. Alternatively, sequences of known function may be recombined to produce modified bacteria having desired properties.

The systems and methodologies of the invention are also useful in analyzing bacterial pathogenic mechanisms. In an exemplary embodiment, a gene can
5   be mutated that results in attenuation of a pathogenic bacterium. Such attenuated bacteria may be used in the preparation of vaccines for use, for example, in humans or animals.

The systems and methodologies of the invention are also useful in *in vivo* cloning applications (*e.g.,* in gap filling applications) and/or marker rescue applications.
10

VI.   Antigens and Vaccines

The recombination methods of the present invention may be used to prepare attenuated live vaccines. The principle behind vaccination is to induce an
15   immune response in the host thus providing protection against subsequent challenge with a pathogen. This may be achieved by inoculation with a live attenuated strain of the pathogen, i.e. a strain having reduced virulence such that it does not cause the disease caused by the virulent pathogen. Typically, attenuation is achieved by mutating genes which are required for virulence/pathogenicity or viability in a host.
20   The recombination methods of the invention may be used to introduce mutations into a bacterium which result in attenuation of that bacterium. The attenuated bacterium can then be used in a vaccine. The bacterium which is attenuated using recombination methods of the invention can contain a non-reverting mutation in at least one gene, for example, one, two, three, four, five or more, which is required for
25   pathogenicity.

The mutations introduced into a bacterium for use in a vaccine generally knock-out the function of the gene completely. This may be achieved either by abolishing synthesis of any polypeptide from the gene or by making a mutation that results in synthesis of non-functional polypeptide. In order to abolish synthesis of a
30   polypeptide, either the entire gene or part of the gene, *e.g.,* its 5'-end, may be replaced using the recombination methods of the invention. Alternatively, the recombination methods of the invention may be used to introduce insertions or deletions into the coding sequence of a gene to create a gene that encodes a non-functional peptide (*e.g.,* polypeptide that contains only the N-terminal sequence of the wild-type protein). The

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recombination methods of the invention may also be used to introduce mutations, *e.g.*, point mutations, into the sequence of a gene to create a gene that encodes a non-functional peptide (*e.g.*, a mutation introducing a stop codon into a gene such that a truncated protein is produced). The bacterium may have mutations in one or more, for
5   example, one, two, three or four genes. The mutations are non-reverting mutations. These are mutations that show essentially no reversion back to the wild-type when the bacterium is used as a vaccine. Such mutations are typically insertions and deletions. Insertions and deletions are preferably large, typically at least 10 nucleotides in length, for example from 10 to 600 nucleotides. Preferably, the whole coding sequence is
10   deleted.

Deletions may be carried out by replacing a target coding sequence with an integrating segment and then subsequently removing the integrating segment as is described above. The bacterium used in the vaccine preferably contains only defined mutations, i.e. mutations which are characterized. It is clearly undesirable to use a
15   bacterium which has uncharacterized mutations in its genome as a vaccine because there would be a risk that the uncharacterized mutations may confer properties on the bacterium that cause undesirable side-effects.

In addition, if the bacterium is to be used in a vaccine and the *exo, bet* and *gam* genes are expressed from a plasmid, it is preferable to remove the plasmid
20   before the bacterium is used in vaccination. There a number of ways of removing plasmids, which are well known to those skilled in the art. For example, the plasmid expressing *exo, bet* and *gam* may be temperature sensitive (ts). Thus, a ts-replicon may be included in the plasmid. The use of plasmids including a ts-replicon allows recombination to be carried out in cultures grown at permissive temperature (*e.g.*, 30°C).
25   Following recombination of genetic material, the growth temperature of the *E.coli* host can be raised to a non-permissive temperature (*e.g.*, 43°C). Under these conditions, the replicon cannot function, and consequently colonies can be isolated that are plasmid-free.

The attenuated bacterium of the invention may be genetically engineered
30   to express an antigen that is not expressed by the native bacterium (a "heterologous antigen"), so that the attenuated bacterium acts as a carrier of the heterologous antigen. The antigen may be from another organism, so that the vaccine provides protection against the other organism. A multivalent vaccine may be produced which not only

provides immunity against the virulent parent of the attenuated bacterium but also provides immunity against the other organism. Furthermore, the attenuated bacterium may be engineered to express more than one heterologous antigen, in which case the heterologous antigens may be from the same or different organisms. The heterologous

5   antigen may be a complete protein or a part of a protein containing an epitope. The antigen may be from a virus, prokaryote or a eukaryote, for example another bacterium, a yeast, a fungus or a eukaryotic parasite. The antigen may be from an extracellular or intracellular protein.

A DNA construct comprising the promoter operably linked to DNA

10   encoding the heterologous antigen may be made and transformed into the attenuated bacterium using conventional techniques. Transformants containing the DNA construct may be selected, for example by screening for a selectable marker on the construct. Bacteria containing the construct may be grown in vitro before being formulated for administration to the host for vaccination purposes. The vaccine may be formulated

15   using known techniques for formulating attenuated bacterial vaccines. The vaccine is advantageously presented for oral administration, for example in a lyophilized encapsulated fond. Such capsules may be provided with an enteric coating comprising, for example, Eudragate "S" (Trade Mark), Eudragate "L" (Trade Mark), cellulose acetate, cellulose phthalate or hydroxypropylmethyl cellulose. These capsules may be

20   used as such, or alternatively, lyophilized material may be reconstituted prior to administration, *e.g.*, as a suspension. Reconstitution is advantageously effected in a buffer at a suitable pH to ensure the viability of the bacteria. In order to protect the attenuated bacteria and the vaccine from gastric acidity, a sodium bicarbonate preparation is advantageously administered before each administration of the vaccine.

25   Alternatively, the vaccine may be prepared for parenteral administration, intranasal administration or intramuscular administration.

The vaccine may be used in the vaccination of a mammalian host, particularly a human host but also an animal host. An infection caused by a microorganism, especially a pathogen, may therefore be prevented by administering an

30   effective dose of a vaccine prepared according to the invention. The dosage employed will ultimately be at the discretion of the physician, but will be dependent on various factors including the size and weight of the host and the type of vaccine formulated.

However, a dosage comprising the oral administration of from 10^7 to 10^{10} bacteria per dose may be convenient for a 70 kg adult human host.

This invention is further illustrated by the following examples which
5 should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application are incorporated herein by reference.

EXAMPLES

10 **General Methodology:**

A.   Strains and Plasmids

Strains used and generated in this study are listed in Table 1. The strain KC5 (EHECΔtir) has been previously cited (Campellone, et al., 2002); its construction is described here. KC12 is an EPEC strain expressing EHEC *tir-cesT-eae*; KC13 is a
15 Δtir::cat-sacB version of KC12; KC26 is a Δtir version of KC12 (Campellone, et al., 2002). Strains expressing chromosomal versions of Tir were generated by electroporating KC13 with linear fragments derived from *ApaLI-XhoI* digestions of pKC17, pKC142, and pKC166 (see Tables 1 & 2). Plasmids pKM154, pTP550, pTP223, pTP806, pTP826, and Tir-expressing plasmids designated pTir-PPP (pKC17)
20 and pTir-HHH$_{NBS}$ (pKC142) have been described previously (Murphy *et al.*, 2000; Semerjian *et al.*, 1989; Poteete and Fenton, 1984; Poteete *et al.*, 1999; Campellone *et al.*, 2002). pAMPts is an ampicillin derivative of pMAK705 (Hamilton *et al.*, 1989). A Cam^R version of pKM201 (pKM200) was also constructed and behaves similarly to pKM201. Plasmids constructed for this study are shown in Table 2.

25   A description of pKM208 and representative predecessors, is as follows:

pKM200   The *Bgl* II fragment from pTP806, containing Ptac-*gam- red* , was ligated to the *Bam* HI site in pMAK705. Temperature- sensitive *red- gam* expressing plasmid; Cam ʀ .

30
pKM201   The *Bgl* II fragment from pTP806, containing Ptac-*gam- red* , was ligated to the *Bam*HIsite in pAMPts. Temperature- sensitive *red- gam* expressing plasmid; Amp ʀ .

35 pKM208   The *Eco* R-*Pst* I *lacI* -containing fragment from pTP550 was treated with T4 DNA polymerase and dNTPs, ligated to *NotI* linkers, cut with

Not I, and ligated to the *NotI* site in pKM201. Temperature- sensitive *red- gam* and *lacI* expressing plasmid; Amp R .

Table 1

Strains used and constructed in this study

Strain	Description	Source or reference
EDL933	EHEC O157:H7 prototype	Riley et al (1983)
TUV93-0	Stx- derivative of EDL933	A. Donohue-Rolfe
JPN15/pMAR7	Amp^R derivative of EPEC E2348/69 O127:H6 prototype	Jerse et al (1990)
Derivatives of EHEC TUV93-0:		
KM45	Δtir-cesT-eae::cat	TUV93-0/ pTP223 x SacI-SphI fragment from pKM185
KM46	Δtir-cesT-eae::cat-sacB	TUV93-0/ pTP223 x SacI-SphI fragment from pKM188
KM47	Δeae::cat	TUV93-0/ pTP223 x SacI-SphI fragment from pKM184
KM48	Δeae::cat-sacB	TUV93-0/ pTP223 x SacI-SphI fragment from pKM187
KM60	Δeae	KM48/ pTP223 x SacI-SphI fragment from pKM181
KM61	Δtir-cesT-eae	KM46/ pTP223 x SacI-SphI fragment from pKM182
KC2	Δtir::cat	TUV93-0/pTP223 x KpnI fragment from pKC2
KC3	Δtir::cat-sacB	TUV93-0/ pTP223 x KpnI fragment from pKC3

KC5	Δtir	KC3/ pTP223 x KpnI fragment from pKC5
KC27	Δtir::cat	TUV93-0/pKM201 x PCR fragment (5KO-H-tir, 3KO-H-tir)
KC30	ΔLEE::cat	TUV93-0/pKM201 x PCR fragment (5KO-H-LEE, 3KO-H-LEE)
KC40	ΔespF::cat	TUV93-0/pKM201 x PCR fragment (5KO-H-espF, 3KO-H-espF)
KM90	ΔlacZ::cat	TUV93-O/pKM201 x PCR fragment (cat13, cat14)
KM94	ΔlacZ::kan	TUV93-O/pKM208 x PCR fragment (kan3, kan4)
KM95	ΔeaeH::kan	TUV93-O/pKM208 x PCR fragment (5KO-H-Island 12, 3KO-H-Island 12)
KM96	ΔZ3025-Z3026::kan	TUV93-O/pKM208 x PCR fragment (5KO-H-Island 77, 3KO-H-Island 77)
KM97	ΔZ3664::kan	TUV93-O/pKM208 x PCR fragment (5KO-H-Island 103, 3KO-H-Island 103)
KM98	ΔhopD-Z4695-yheA::kan	TUV93-O/pKM208 x PCR fragment (5KO-H-Island 130-131, 3KO-H-Island 130-131)
KM99	ΔZ58155-Z5816::kan	TUV93-O/pKM208 x PCR fragment (5KO-H-Island 169, 3KO-H-Island 169)

Derivatives of EPEC JPN15/pMAR7

| KC21 | ΔLEE::cat | JPN15/pMAR7/ pTP223 x PCR fragment (3KO-P-LEE, 5KO-P-LEE) |

Table 2

Plasmids constructed for this study

pKC2 Regions upstream and downstrem of EHEC *tir*, generated by PCR using primer pairs H-TirU/H-TirN and H-TirC/H-TirD, respectively, were fused in a second stage PCR reaction to the *cat* gene from pTP883 to generate a Δ*tir::cat* DNA fragment, which was cut with *Kpn*I and ligated to the *Kpn*I site of pUC19 (Δ*tir::cat* plasmid).

pKC3 The *Not*I fragment containing *cat-sacB* from pKM154 was ligated to the *Not*I site of pKC2 (Δ*tir::cat-sacB* plasmid).

pKC5 pKC2 was digestedwith *Not*I and the backbone was religated, generating an EHEC fragment which replaces codons 24-543 of *tir* with the *Not*I-containing sequence GCGGCCGCG (in-frame Δ*tir* plasmid).

pKM181 The PCR product containing the 1.5 kb region upstream of *eae*, generated by primers pairs EaeU/EaeN, was digested with *Sac*I and *Not*I and ligated to the *Sac*I-*Not*I fragment from pKM182, containing the 1.5 kb downstream region of *eae*, generating an EHEC fragment which replaces codons 4-932 of *eae* with the *Not*I-containing sequence GCGGCCGCA (in-frame Δ*eae* plasmid).

pKM182 The 1.5 kb regions upstream and downstrem of the EHEC *tir-cesT-eae* region were generated by PCR using primers pairs TirU/TirN and EaeC/EaeD, respectively. The upstream fragment was cut with *Sac*I and *Not*I and the downstream fragment was cut with *Sph*I and *Not*I. These fragments were cloned in a 3-piece ligation into

the *SacI-SphI* backbone of pUC19, generating an EHEC fragment that fuses codon 3 of *tir* to codon 933 of *eae* via an in-frame *NotI*-containing sequence GCGGCCGCA (in-frame Δ*tir-cesT-eae* plasmid).

pKM184   The *ApaI-NheI cat*-containing fragment from pTP826 was treated with T4 polymerase and dNTPs, ligated to *NotI* linkers, cut with *NotI*, and ligated to the *NotI* site of pKM181 (Δ*eae::cat* plasmid).

pKM185   The *ApaI-NheI cat*-containing fragment from pTP826 was treated with T4 polymerase and dNTPs, ligated to *NotI* linkers, cut with *NotI*, and ligated to the *NotI* site of pKM182 (Δ*tir-cesT-eae::cat* plasmid)

pKM187   The *NotI* fragment containing *cat-sacB* from pKM154 was ligated to the *NotI* site of pKM181 (Δ*eae::cat sacB* plasmid).

pKM188   The *NotI* fragment containing *cat-sacB* from pKM154 was ligated to the *NotI* site of pKM182 (Δ*tir-cesT-eae::cat sacB* plasmid).

pKM200   The *BglII* fragment from pTP806, containing Ptac-*gam-red*, was ligated to the *BamHI* site in pMAK705. Temperature-sensitive *red-gam* expressing plasmid; Cam^R.

pKM201   The *BglII* fragment from pTP806, containing Ptac-*gam-red*, was ligated to the *BamHI* site in pAMPts. Temperature-sensitive *red-gam* expressing plasmid; Amp^R.

pKM208   The *EcoR-PstI lacI*-containing fragment from pTP550 was treated with T4 DNA polymerase and dNTPs, ligated to *NotI* linkers, cut with *NotI*, and ligated to the *NotI* site in pKM201. Temperature-sensitive *red-gam* and *lacI* expressing plasmid; Amp^R.

Table 3

Primers used for this study

Generation of long homology plasmid substrates:

TirU   ATCATCGAGCTCACCGAGCAGTTCTCGATTGCTATT (*SacI*)   **(SEQ ID NO:1)**

TirN   TTAGACGAATGCGGCCGCAATAGGCATAAATATCTCCTTTT (*NotI*)   **(SEQ ID NO:2)**

TirC   ATGCCTATTGCGGCCGCATTCGTCTAAATATATCCATAATCA (*NotI*)   **(SEQ ID NO:3)**

TirD   ATCATCGCATGCCACCAGAAAAATCCTGATCAATGA (*SphI*)   **(SEQ ID NO:4)**

EaeU   ATCATCGAGCTCGGAAATGCGATTCCGTCA (*SacI*)   **(SEQ ID NO:5)**

EaeN   TTATTCTACTGCGGCCGCAGTAATCATGTTATGGCTCCACCA (*NotI*)   **(SEQ ID NO:6)**

EaeC   ATGATTACTGCGGCCGCAGTAGAATAATTCCATAACCACCCC (*NotI*) **(SEQ ID NO:7)**

EaeD   ATCATCGCATGCTAAAACTTCTCAATGGTGCGATGC (*SphI*)   **(SEQ ID NO:8)**

H-tirU   ATCATCGGTACCGCACGTCAGTTGCTCTTCAAGAG (*KpnI*)   **(SEQ ID NO:9)**

H-tirN   TGCCGATCAACGTCTCATGCGGCCGCAGGTAATGGAGGTGCAGGAGGAAT (*NotI*)   **(SEQ ID NO:10)**

H-tirC   AATGGCAGAAATTCGAAAGCGGCCGCGAATACTTCGAATAACCCACCAGC (*NotI*)   **(SEQ ID NO:11)**

H-tirD   TCATCAGGTACCTCGGTCATGTGTGCTTTGGTCACG (*KpnI*) **(SEQ ID NO:12)**

Generation of PCR substrates for one-step gene deletion:

5KO-H-lacZ   GACGGGTTGTTACTCGCTCACATTTAATGTTGATGAAAGCGGCGGCCGCATGAGACGTTGA **(SEQ ID NO:13)**

3KO-H-lacZ   AAGAAAGCCTGACTGGGCGGTTAAAATTGCCAACGCTTATTAGCGGGCCGCTTTCGAATTCT **(SEQ ID NO:14)**

5KO-H-EspF   ATGCTTAATGGAATTAGTAACGCTGCTTCTACACTAGGGCGGCAGCTTGTAGCGGCCGCATGAGACGTTGAT **(SEQ ID NO:15)**

3KO-H-EspF   TTACCCTTCTTCGATTGCTCATAGGCAGCTAAATGATCTTTAATGCCTG GCGGCCGCTTTCGAATTCTGC **(SEQ ID NO:16)**

5KO-H-tir   ATGCCTATTGGTAATCTTGGTCATAATCCCAATGTGAATAATTCAATTCCTCCTGCACCTC GCGGCCGCATGAGACGTTGA **(SEQ ID NO:17)**

3KO-H-tir   GATATATTAGACGAAACGATGGGATCCCGGCGCTGGTGGGTTATTCGAAGTATTCACA GCGGCCGCTTTCGAATTCT **(SEQ ID NO:18)**

Generation of PCR substrates for one-step island deletion:

5KO-H-LEE   TTTCTGTTATCATTACTGCCAATATTTGTTGTTATTGGTACTTCATTCCTGAAAGCGGCCGCATGAGACGTTGAT **(SEQ ID NO:19)**

3KO-H-LEE   AAAGCTGTCGAAATATTAATCGCGATAAATGATATCCACCACAACTGTTGGTAGTGCGGCCGCTTTCGAATTCTGC **(SEQ ID NO:20)**

5KO-P-LEE   AAGTATTTATTGAATTCATTTAAAGATAATTATCTTAGCATTATTCAGGCGGGCCGCAT GAGACGTTGAT (SEQ ID NO:21)

3KO-P-LEE   TGTTCTTCTGATATCCAGAAACGCCCCTCATAGCCCGAGTATGTCAACGTGCGGCCGCT TTCGAATTTCTGC (SEQ ID NO:22)

ΔIsland 12A   CTGACACTGAGCGGCCGGGGCATAAGCCAGGGCAAGAGCGGTGAATCTCTGATGTTACATTGC (SEQ ID NO:23)

ΔIsland 12B   CCTCTTTCCGCTATGAAGGTGAGTGGGAGCACTACCCTGATTCAACTCAGCAAAAGTTCG (SEQ ID NO:24)

ΔIsland 77A   AGCCAGGTCAACAGGTCAGTATGGGAAGGGCGAACAACTCGAATCTCTGATGTTACATTGC (SEQ ID NO:25)

ΔIsland 77B   TGTTGTTAATGACATCCGATCTCACCGCGTGGGCATGGATTCAACTCAGCAAAAGTTCG (SEQ ID NO:26)

ΔIsland 103A   TTGACATCCTCCACGCCCTGAATGACGAGGATCCCTGCTAAATCTCTGATGTTACATTGC (SEQ ID NO:27)

ΔIsland 103B   GGTGGCGCCGTAAAACCCGTCCTTCAGGGGCGGGGATATAATTCAACTCAGCAAAAGTTCG (SEQ ID NO:28)

ΔIsland 130-131A   GCAAATCTGAGCCTGACGCAAGCATCGGGCAGAAATTAATAATCTCTGATGTTACATTGC (SEQ ID NO:29)

ΔIsland 130-131B   TGCCCGTAATTTGAGCTCGAAATATTTAGTCGTAATTTGTTCAACTCAGCAAAAGTTCG (SEQ ID NO:30)

ΔIsland 169A   CCGGTGCGCCGTAAAACCCCGTCCTTCAGGGCGGGGATATAATCTCTGATGTTACATTGC
(SEQ ID NO:31)

ΔIsland 169B   TTGACATCCTCCAAGCCCTGAAGGACGTGGATCCCTGCTATTCAACTCAGCAAAAGTTCG
(SEQ ID NO:32)

ΔIsland 169E   CCGGTGCGCCGTAAAACCCCGTCCTTCAGGGCGGGGATATTTCAACTCAGCAAAAGTTCG
(SEQ ID NO:33)

ΔIsland 169F   TTGACATCCTCCAAGCCCTGAAGGACGTGGATCCCTGCTAAATCTCTGATGTTACATTGC
(SEQ ID NO:34)

B.   Electroporation and gene replacement protocol

A single fresh colony of EHEC or EPEC was placed into 20 ml of LB plus 15 μg ml^{-1} tetracycline (for pTP223) or 100 μg ml^{-1} ampicillin (for pKM201 and pKM208) and shaken at 30°C in an 125 ml flask. At ~10^7 cells/ml, IPTG was added to a

5   final concentration of 1 mM. When the culture reached a density of 0.5 - 1 x 10^8, the cells were heat shocked for 15 minutes by swirling at 42°C, transferred to an ice-water bath for 10 min with swirling, then collected by centrifugation. The cells were resuspended in 1 ml of ice-cold 20% glycerol - 1mM MOPS (unbuffered), transferred to a 1.5 ml sterile eppendorf tube, and spun in a microfuge for 30 seconds (moderate

10   speed). The supernatant was removed; the cells were resuspended in the same buffer and recentrifuged. This step was repeated and the cells were finally resuspended in 90-100 μl of ice-cold 20% glycerol - 1 mM MOPS.

Electroporation cuvettes (Biorad) were cooled in an ice-water bath for at least 10 minutes prior to use. DNA samples contained either 0.1 - 0.5 μg of purified

15   DNA fragments or 0.2 - 10.0 μg of plasmid digests in TE or water. A 50 μl sample of cells was mixed with 1-5 μl of DNA, transferred to the electroporation cuvette, and incubated on ice for 1 minute. The cuvette was thoroughly but quickly dried and the cells were shocked as described previously (Murphy, 1998). Following electroporation, the cells were recovered by suspension in 0.3 ml LB, diluted in 2.7 ml LB, grown by

20   rolling at 37° for 1.5 hour, and plated on LB plates containing either 10-15 μg ml^{-1} chloramphenicol or 20 μg ml^{-1} kanamycin. (Growing for less that one hour with kanamycin markers drastically reduced the recovery of recombinants). Alternatively, though usually not required, the cell cultures were grown overnight before plating. After overnight growth, drug-resistant transformants of EHEC and EPEC were

25   restreaked on LB plates containing the appropriate drug.

In some of the early experiments, ice-cold water was used instead of 20% glycerol - 1 mM MOPS for resuspending the cells. However, it was found that the glycerol/MOPS buffer improved the electroporation survivor rate for EHEC (and thus recovery of recombinants), as was reported for electroporation of *Pseudomonas*

30   *aeruginosa* (Farinha and Kropinski, 1990). In some experiments, transformants were also checked for tetracycline (pTP223) or ampicillin (pKM201 and pKM208) sensitivity indicating loss of the Red-producing plasmid (if desired). These Red-producing

plasmids were lost spontaneously at relatively high frequency following growth and electroporation, *i.e.,* selection at 42 degrees for loss of the temp-sensitive pKM208 replicon was usually not required. Various numbers of transformants that successfully restreaked on to fresh drug plates were analyzed by PCR to verify chromosomal

5   replacement of the target gene(s), as described in Figure 1. The selection for Suc^R-Cam^R transformants (containing precise in-frame deletions) was done as described previously for *E. coli* K-12 (Murphy *et al.,* 2000).

C.   *Infections and immunoflourescence microscopy*

HeLa cells were cultured in DMEM plus 10% fetal bovine serum, 100 U

10   ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 2 mM L-glutamine at 37°C in 5% CO_2. Prior to infections, EPEC were cultured in DMEM + 100 mM HEPES pH 7.4 in 5% CO_2, growth conditions previously shown to maximize type III secretion. HeLa cells grown on 12 mm glass coverslips were infected in DMEM plus 3% fetal bovine serum, 20 mM HEPES pH 7.4, and 2 mM L-glutamine at 37°C in 5% CO_2 for five hours and

15   processed as described previously (Campellone *et al.,* 2002). Monolayers were stained with a 1:500 dilution of anti-HA mAB HA.11 (Covance) for 30 minutes followed by an additional 30 minute staining with 1 μg ml^{-1} TRITC-phalloidin (Sigma), 1 ug ml^{-1} DAPI, and a 1:200 dilution of Alexa488-conjugated goat anti-mouse IgG (Molecular Probes).

20   ### D.   *Infections and immunoblotting*

HeLa cells and bacteria were cultured as described above. 90% confluent HeLa cell monolayers grown in 6-well plates were infected with approximately 10^8 EPEC per well for 3.5 hours. Bacteria were then washed 3 times with PBS, killed by treatment for an additional 0.75 hours with 50 μg ml^{-1} gentamicin in infection media,

25   and washed 5 times with PBS. Cells were collected by treatment with PBS + 2mM EDTA, washed once with PBS, lysed in lysis buffer (50 mM HEPES pH 7.4, 50 mM NaCl, 1 mM Na_3VO_4, 10 μg ml^{-1} peptstatin, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, and 100 μg ml^{-1} PMSF) and processed for western blotting. Infected HeLa lysate samples were boiled in SDS-PAGE loading buffer, electrophoresed, and transferred to

30   PVDF membranes. The membranes were probed with anti-HA antiserum (1:1000) as described previously (Campellone, et al., 2002). N-WASP and OmpA served as loading controls for cells and bacteria, respectively, and were stained with rabbit anti-rat N-

WASP antiserum (1:1000) and anti-OmpA antiserum (1:1000) and visualized with alkaline-phosphatase conjugated anti-rabbit antiserum.

Introduction to Examples 1-7

5   Pathogenic *E. coli* species should be amenable to the use of λ Red for chromosomal engineering. Enterohemorrhagic *E. coli* O157:H7 (EHEC) and enteropathogenic *E. coli* (EPEC) are members of the attaching and effacing (AE) family of enteric pathogens [21, 22]. These pathogens bind tightly to the intestinal epithelium and cause localized effacement of micovilli, followed by alteration of the cytoskeleton

10   beneath sites of bacterial attachment. Some labs have used λ Red to promote gene knockouts in EPEC by recombination between linear plasmid DNA fragments containing long regions of homology (~ 0.5 - 1 kb) and the EPEC chromosome [23-25]. In addition, a few reports have utilized PCR substrates containing short regions of homology to perform gene replacement in EHEC [26-28], the latter report employing

15   plasmids and protocols described herein. None of these reports, however, described the frequency of recombinant formation or the reproducibility of Red-promoted PCR-mediated recombination in EHEC at multiple loci. Indeed, initial attempts by the instant inventors to employ λ Red for PCR-mediated gene replacement at various loci in EHEC in EPEC were met with sporadic success, similar to the limited success seen with Red-

20   promoted short homology recombination in Y. *pseudotuberculosis*. These difficulties prompted the inventors to examine more closely the methodologies of λ Red promoted PCR-mediated gene replacement, especially in regard to optimizing its use in EHEC and EPEC.

Examples 1-7 demonstrate that expression of bacteriophage λ *red* and

25   *gam* recombination functions in enterohemorrhagic *E. coli* O157:H7 (EHEC) and enteropathogenic *E. coli* (EPEC) promotes efficient recombination with linear DNA substrates in these pathogens. This technology has been used to generate marked and unmarked deletions of known virulence genes *eae* (intimin) and *tir* (translocated intimin receptor). In addition, several EHEC/EPEC *tir* hybrids have been crossed onto the

EPEC chromosome at the endogenous *tir* locus. A hybrid Tir that contains an EPEC-Tir derived Nck-binding site in the context of an otherwise EHEC Tir promoted wild type looking pedestals when overexpressed from a low-copy number plasmid, but formed extended pedestals when expressed at normal levels from its chromosomal location.

5 The suppression of this phenotype when this Tir molecule is expressed from a low copy number plasmid highlights the utility of a genetic technique that allows one to express mutant genes from their chromosomal locations. Finally, using the λ Red system, five O157-specific islands (O-islands) of EHEC were easily and precisely deleted from the chromosome by electroporation with PCR-generated substrates containing drug markers

10 flanked with 40 bp of target DNA. PCR-mediated λ Red-promoted recombination was also successful in EPEC.

The instant inventors have found conditions that allowed PCR-mediated recombinants to be reproducibly obtained using λ Red recombination in EHEC and EPEC, guidelines that can be applied to the use of Red in other pathogenic bacteria.

15 These steps include the use of an optimal buffer for the preparation of electrocompetent cells, a heat-shock step that induces higher frequencies of gene replacement, and proper positioning of the drug marker within the recombinant PCR substrate. As demonstration of the utility of this technology, five EHEC O-islands were easily deleted from the EHEC chromosome by simple electroporation with PCR-generated substrates. The importance

20 of limiting expression of Red functions during growth of EHEC was noted, as extended expression of the recombination functions induces a 10-fold increase in spontaneous mutagenesis. Gene replacement frequencies generated by various treatments of plasmid substrates to construct marked and precise deletions of EHEC *eae*, *tir*, and the *eae-cesT-tir* operon, are presented.

25

Example 1: Red-mediated recombination in Pathogenic Bacteria Using Long homology recombination (LHR) substrates (1-2 kb)

The present inventor has previously shown that the multi-copy plasmid pTP223 supports λ Red-mediated recombination with long substrates (drug markers

30 flanked by ~ 1 kb of target DNA; Murphy, 1998; Murphy *et al.*, 2000). This plasmid

expresses both λ Red and the anti-RecBCD function, Gam, under control of the Plac promoter, as well as the *lacI* repressor gene from its own promoter (Poteete and Fenton, 1984). Plasmid substrates were constructed that contained the *cat* gene (conferring resistance to chloramphenicol) flanked by upstream and downstream regions of *tir, eae,*

5   or the *tir-cesT-eae* operon (*cesT* encodes a chaperone for Tir). Linear DNA recombination substrates (*i.e.*, the *cat* gene flanked by 1.5 kb of target DNA) were generated from these plasmids by digestion with restriction enzymes (see Figure 1A and Tables 1 & 2 for details). EHEC cells harboring pTP223 were electroporated with the restriction digests, or gel-purified linear fragments containing the marked deletions, and

10   plated on LB plates containing chloramphenicol. Among the chloramphenicol resistant colonies, potential chromosomal substitutions were distinguished from simple plasmid transformants by their sensitivity to ampicillin (*bla* gene carried within the pUC19 plasmid backbone). Results of a number of these experiments are shown in Table 4.

      In all cases, Δ*eae,* Δ*tir,* or Δ*eae-cesT-tir* was easily generated with these

15   plasmid substrates, generating as few as 2 and as many as 1000 gene replacements per experiment, depending on the nature and amount of the transforming DNA. These genes were replaced with either the *cat* drug marker (Table 4, lines 1, 2, 4-9) or a *cat-sacB* cassette (Table 4, lines 3, 10 and 11). The latter substitutions generated strains that were subsequently used to generate in-frame, precise deletions (see Table 5). All gene

20   replacements were verified by PCR analysis (as described in Figure 1 - data not shown). Overall, the following observations were evident. Electroporation of simple plasmid digests resulted in a high number of plasmid transformants (*i.e.*, Cam^R-Amp^R, see Table 4, lines 1-3), due to incomplete digestion or religation of the plasmid substrate *in vivo*. A higher number of potential gene replacement transformants (*i.e.*, Cam^R- Amp^S colonies)

25   could be obtained by further digestion of the plasmid with a backbone-specific restriction enzyme, *ApaL*I (see Table 4, lines 4-7), or by gel purification of the linear DNA recombination substrate (Table 4, lines 8-11).

      Surprisingly, the Red-producing plasmid pTP223 is spontaneously lost during this process (between 10-50% of the transformants are Tet^S). Thus, a separate

30   step to cure the recombinant of the Red plasmid is not required. These experiments show that marked and precise deletions can be easily generated in EHEC without the need to form and resolve plasmid co-integrates. This procedure can likewise be used to generate deletions, for example, at the *recC* and *dam* loci in EHEC.

Table 4

Gene replacements in the *tir-cesT-eae* region of enterohemorrhagic
E. coli 0157:H7 using long homology-containing substrates

Exp #	Substrate preparation	Amount of DNA	Genotype of linear fragment	Total Transformants	Percent transformants Amp sensitive	Recombinants verified by PCR (# verified/# tested)
Plasmid digests:						
1.	restriction digest pKM184 (*SacI, SphI*)	2 µg	Δ*eae::cat*	>500	11% (4/35)	1/1
2.	restriction digest pKM185 (*SacI, SphI*)	2 µg	Δ*eae-cesT-tir::cat*	103	2.6% (2/76)	1/1
3.	restriction digest pKM 187 (*SacI, SphI*)	1 µg	Δ*eae::cat sacB*	>500	2.8% (1/35)	1/1
Plasmid digests including backbone:						
4.	restriction digest pKM184 (*SacI, SphI, ApaLI*)	0.5 µg	Δ*eae::cat*	52	57% (4/7)	NT
5.	restriction digest pKM184 (*SacI, SphI, ApaLI*)	10 µg	Δ*eae::cat*	> 500	95% (143/151)	10/10
6.	restriction digest pKM185 (*SacI, SphI, ApaLI*)	2 µg	Δ*eae-cesT-tir::cat*	30	56% (9/16)	9/10
7.	restriction digest pKM185 (*SacI, SphI, ApaLI*)	2 µg	Δ*eae-cesT-tir::cat*	36	50% (9/18)	7/9
Gel-purified fragments:						
8.	purified *SacI-SphI* fragment from pKM185	0.5 µg	Δ*eae-cesT-tir::cat*	225	100% (20/20)	1/1
9.	purified *KpnI* fragment from pKC2	0.1 µg	Δ *tir::cat*	6	NT	6/6

Table 5

Generation of EHEC O157:H7 unmarked deletions of *eae* and *tir*

Exp	Substrate Preparation	Amount of DNA	Genotype of linear fragment	Genotype of recipient		Percent $Suc^R Cam^S$ transformants[a]	Recombinants verified by PCR (# verified/# tested)
10.	purified *SacI-SphI* fragment from pKM188	0.6 μg		Δ*eae cesT tir*::*cat sacB*	273	100% (40/40)	1/1
11.	purified *KpnI* fragment from pKC3	0.05 μg		Δ*tir*::*cat sacB*	2	NT	2/2
1.	Digest of pKM182 (*SacI, SphI*)	2 μg	Δ*eae-cesT-tir*	Δ*eae-cesT-tir*::*cat-sacB*		94% (94/100)	6/6
2.	Digest of pKM181 (*SacI, SphI*)	2 μg	Δ*eae*	Δ*eae*::*cat-sacB*		88% (88/100)	6/7
3.	Purified *KpnI* fragment from pKC5	0.1 μg	Δ*tir*	Δ*tir*::*cat-sacB*		10.5% (2/19)	2/2

a) The selection for Suc^R-Cam^S transformants (containing precise in-frame deletions) was performed as described previously for *E. coli* K-12 (Murphy et al, 2000).

43

Example 2: Red-mediated recombination does not compromise infectious processes of the pathogenic bacteria EHEC and EPEC

To determine if Red-mediated recombination induced any deleterious

5   effects on EPEC or EHEC strains, the abilities of several strains to form actin pedestals
on cultured mammalian cells were tested. Strains EHECΔtir and EPECΔtir (KC5 and
KC14, respectively) were generated by Red-mediated recombination using pTP223 (see
Table 1). As expected, due to the requirement of Tir in actin signaling, neither of these
strains formed pedestals on infected HeLa cells, but were capable of being functionally

10   complemented for pedestal formation by plasmid encoded Tir (Campellone, *et al.*,
2002). These results indicate that no obvious ectopic mutations arose in these two
different Red-treated strains to prevent the highly coordinated tasks of bacterial binding
and type III translocation of effector proteins.

15   ## Example 3: Benefits of using Red-mediated recombination in functional genomics applications

This Example demonstrates at least one benefit of expressing proteins
(*e.g.*, mutant proteins) from a chromosomal location versus overexpressing from a
plasmid. In this Example, it is first demonstrated that proteins are expressed at lower

20   (*i.e.*, normal) levels when expressed from a chromosomal location, as compared to
artificially high levels expressed from a comparable plasmid. Expression at normal
levels unmasked a mutant phenotype that was not observed in the overexpression
situation. It appears that overexpression of mutant protein rescued, and thus masked, the
mutant phenotype. This experiment demonstrates the benefit of expressing mutant

25   proteins at a normal level (*i.e.*, from a chromosomal location) when performing
functional genomics studies.

Expression levels and translocation into host cells of proteins expressed from a
chromosomal location as compared to plasmid-expressed proteins

30   One advantage of being able to couple the efficiency of Red-mediated
recombination with the counterselectable marker *sacB* is the ability to cross DNA
fragments containing molecular alterations onto endogenous chromosomal loci. These

mutants can then be tested for function when expressed at normal levels, thereby avoiding any potential plasmid-borne overexpression artifacts.

Recently, several laboratories have observed that the EHEC Tir molecule does not function for actin pedestal formation when expressed in EPEC (Campellone, *et al.*, 2002; DeVinney, *et al.*, 1999; Kenny, 2001). A twelve amino acid sequence of the EPEC Tir molecule required for actin signaling and binding to the host adaptor protein Nck was identified in the context of plasmid-derived EHEC/EPEC Tir chimeras expressed in an EPEC strain harboring EHEC intimin (Campellone, *et al.*, 2002). The plasmid-derived expression levels of some of these Tir molecules was compared with identical Tirs which were crossed onto the EPEC chromosome (*via* Red-mediated recombination). In particular, HA-tagged wild type EPEC Tir (Tir-PPP) and two EHEC/EPEC Tir chimeras (Fig. 2A) were examined for levels of Tir expression and translocation into host cells. One chimera possessed the N- and C-terminal cytoplasmic domains of EPEC Tir with the central extracellular (intimin-binding) domain of EHEC Tir (Tir-PHP), and one was entirely composed of EHEC Tir with the exception of an EPEC-Tir derived Nck-binding site (NBS) incorporated into its C-terminal cytoplasmic domain (Tir-HHH$_{NBS}$) (Fig. 2A). HeLa cell monolayers were infected with EPEC strains expressing these three versions of Tir either from the endogenous Tir locus or from a low copy plasmid, with expression driven by an identical EPEC Tir promoter in each case. Non-intimately associated bacteria were killed with gentamicin, and the remaining infected HeLa cells were collected, processed for SDS-PAGE, and immunoblotted for Tir. Tir residing in the bacterial cytoplasm typically migrates at approximately 78kDa, while Tir which has been translocated into host cells is modified by serine/threonine phosphorylation and migrates at 90kDa (DeVinney, et al., 1999; Kenny *et al.*, 1997).

Examination of Tir immunoblots indicated that for each of the three Tir molecules examined, the bacterially-associated 78kDa form was expressed at higher levels when encoded on a low copy number plasmid (Fig. 2B). Similarly, strains expressing Tir from plasmids translocated more Tir into mammalian cells, as evidenced by increases in levels of the ~90kDa form of Tir (Fig. 2B).

Chromosomal expression of a mutant gene (the EHEC/EPEC Tir chimera) reveals a mutant phenotype (the long-pedestal phenotype)

To determine whether these differences in expression and translocation levels of Tir had any consequences on actin signaling and pedestal formation, EPEC

5   strains expressing Tir-PPP, Tir-PHP, or Tir-HHH$_{NBS}$ were examined for differences in pedestal structures. Wild type EPEC Tir-PPP and chimeric Tir-PHP exhibited similar pedestal formations whether they were delivered at endogenous chromosomal levels or at higher plasmid-driven levels (Fig. 3A-B). In contrast, chimeric Tir-HHH$_{NBS}$, which contains EPEC Tir Nck-binding site incorporated into an otherwise EHEC Tir, displayed

10   striking morphological differences in pedestals depending upon the location of the *tir* gene. EPEC delivering higher levels of Tir-HHH$_{NBS}$ due to plasmid-derived expression generally formed pedestals similar to bacteria expressing wild type EPEC Tir (Fig. 3A). But upon close inspection, a small percentage of these bacteria formed pedestals which appeared to be increased in length (data not shown). However, when Tir-HHH$_{NBS}$ was

15   expressed from its chromosomal locus, bacteria generated a large number of pedestals of increased lengths (Fig. 3B).

These results suggest that chimeric Tir-HHH$_{NBS}$ is partially defective for pedestal formation, since the morphologies of these pedestals are radically different · from wild type (shorter) pedestals, and that this defect can be overcome by

20   overexpression of the chimera. The difference in pedestal formation associated with this Tir chimera can likely be attributed to differences in the N- or C-terminal cytoplasmic domains of the EHEC and EPEC Tir homologues, since chimeric Tir-PHP (which has the EPEC Tir cytoplasmic domains) forms pedestals of normal appearance on HeLa cells (Fig. 3A-B).

25

Example 4: Red-mediated recombination in Pathogenic Bacteria Using Short homology recombination (SHR) substrates (40-60 bp of homology)

Red-promoted gene replacement with the long homology substrates described above required cloning of the marked deletions or substitutions. Despite this

30   restriction, this method of chomosomal engineering can be useful in certain situations (see above, and Discussion). However, a simpler method of generating gene knock-outs in *E. coli* K-12 (and *Salmonella* strains) involves electroporation of PCR products containing short regions (40 bp) of flanking homology to the target gene into λ Red and

Gam-producing bacteria. This strategy has been shown to promote high efficiency gene replacement with such substrates in *E. coli* K-12 (Datsenko and Wanner, 2000; Yu *et al.*, 2000) as well as *Salmonella enterica* (see Introduction). Typically, PCR is performed with two primers (60 mers) which contain 20 bases at the 3' ends to amplify

5 a drug marker. In addition, the primers contain 40 bases at the 5' ends that are complimentary to either the N or C-terminal regions of the target gene (see Figure 1B). The PCR product, which has the drug marker flanked by 40 bases upstream and downstream of the targeted gene of interest, is simply electroporated into Red + Gam producing *E. coli* or *Salmonella.*

10 While pTP223 promotes gene replacement efficiently with substrates containing long regions of homology to the target gene (Murphy, 1998), PCR substrates containing short regions of homology (40 bp) recombine at very low frequency [Datsenko, 2000 #20; Murphy, unpublished observations]. It has been noted that expression of *red* from the chromosome, or low copy number plasmids, is better suited

15 for Red-mediated recombination relative to multi-copy plasmids (Murphy, 1998; Datsenko and Wanner, 2000; Yu *et al.*, 2000). It is assumed that since Red induces the rolling circle mode of replication in medium or high copy number plasmids (Poteete *et al.*, 1988), linear multimers of the plasmid are generated that may compete with the electroporated substrates for the Red recombination functions. This reasoning explains

20 why pTP223, while expressing high levels of Red and Gam, is not optimal for Red-promoted gene replacement in *E. coli* K-12 (especially with short homology substrates).

In contrast, studies by the instant inventor with Δ*recBCD::gam-red* chromosomal substitutions (expressing *red* from either P_{lac} or the stronger P_{tac} promoter) have shown that Red-promoted recombination with short-homology substrates requires

25 higher level expression of the *red* functions relative to long-homology substrates (unpublished observations). A construct that meets both these requirements (high expression from a single or low copy number replisome) is plasmid pKD46, described by Datsenko and Wanner (Datsenko and Wanner, 2000) which uses the pBAD promoter to express *red* and *gam* from a low copy number temperature-sensitive replicon. A similar

30 plasmid, pKM201, was constructed except that *gam* and *red* are driven by the Ptac promoter. A variation of pKM201 was constructed which expresses the *lacI* repressor gene (pKM208), in order to keep expression of *red* and *gam* under tight control prior to

IPTG induction. In anticipation of the requirement to easily remove these plasmids after gene replacement, both plasmids contain temperature-sensitive origins of replication.

EHEC containing pTP223, pKM201, or pKM208 were electroporated with a PCR substrate containing the *kan* gene flanked by 40 bases of N- and C-terminal

5   regions of the *lacZ* gene. As expected, plasmid pTP223 was unable to efficiently promote short-homology recombination in EHEC (data not shown; however, see results with EPEC below). On the contrary, low copy number plasmids pKM201 and pKM208 were able to promote short homology recombination in EHEC at the *lacZ* locus. Recombinants were detected as white Kan^R colonies on LB-kan plates containing X-gal

10   and IPTG. Cells harboring LacI-expressing pKM208 required prior induction with IPTG for efficient recombination (see below); cells containing pKM201 did not require IPTG addition (due to P_{tac} leakiness in the absence of over-expressed LacI).

In five separate experiments, Red expression from pKM208 produced gene replacements at a rate between 70-600 recombinants per 10^8 cell survivor (total

15   number of recombinants varied from 750-3000). From one of these experiments, ten out of ten white Kan^R transformants tested positive for gene replacement by the PCR method described in Figure 1B (data not shown). In addition, a PCR fragment containing a *lacZ* deletion with a *cat* insertion worked as well as the one described above using *kan*. It is noteworthy, however, that while the Δ*lacZ::kan* allele yielded recombinants at a

20   frequency 0.7-6 x 10^{-6} per survivor in EHEC containing pKM208, the same PCR fragment in *E. coli* K-12 containing pKM208 yielded recombinants at a frequency of 10^{-4} per survivor (data not shown). The sequences used in targeting Δ*lacZ* to the *E. coli* K-12 and EHEC chromosomes are identical. The reason for this lower frequency is not known, but may be due to an EHEC-specific restriction system(s) or lower rates of DNA

25   uptake following electroporation; other possibilities are considered in the Discussion section.

Gene replacements using short homology substrates was also performed in EHEC containing pKM201 at the *tir* and *espF* loci within the LEE, though frequencies of gene replacement at these sites (in repeated experiments) were lower than that seen with

30   the *lacZ* substrate described above, and usually ranged from 0-20 recombinants per 10^8 survivor. However, this lower frequency of gene replacement at alternative loci relative to *lacZ* was also observed in *E. coli* K-12 (unpublished observations). Thus, the *lacZ* region may be a hotspot for gene replacement, perhaps the result of stable expression of

the drug marker following integration at this particular locus (see below). Nonetheless, Red-promoted PCR-mediated gene replacement was successful with both *tir* and *espF*.

To assess the overall usefulness of Red-promoted PCR-mediated gene replacement in EHEC, five O157-specific islands (O-islands) in the EHEC chromosome

5 were targeted for deletion; these O-islands are not present in *E. coli* K-12 (Perna *et al.*, 2001). PCR substrates containing the *kan* gene flanked by 40 base pairs of DNA bordering O- islands #12, #77, #103, #130-131 and #169 (Table 6) were electroporated into EHEC containing pKM208. These islands were targeted because they occupy different regions of the chromosome, are of moderate size (733-4253 base pairs in

10 length), and encode either putative virulence factors or unknown proteins. In the first attempt, all five islands were successfully deleted (see Table 6), though there was variability in the frequency of island replacement. Deletion of O-islands #130-131 occurred at a frequency similar to that seen with *lacZ* gene (~100 kan^R transformants per 10^8 cell survivors), while the others showed rates ranging from 10-50 fold lower. Thus, λ

15 Red is able to promote efficient short homology recombination with the EHEC chromosome, though at a reduced frequency relative to that seen in *E. coli* K-12.

The islands selected above ranged from 733-4253 base pairs in length. To determine if any restrictions could be placed on the amount of DNA deleted by Red-promoted recombination, a PCR product was generated containing the *cat* gene flanked

20 by regions upstream and downstream of an internal section of O-island #148, which contains the locus of enterocyte effacement (LEE). Electroporation with this PCR fragment, designed to delete 9 kb of genes encoding the type III secretion apparatus (see Table 1, strain KC30) generated recombinants at a frequency of 20 per 10^8 survivors, similar to other deletion frequencies. Thus, both small and large regions of the EHEC

25 chromosome can be deleted in one step using Red-mediated recombination (as has been seen with *E. coli* K-12). Indeed, an additional 15 islands of EHEC of various sizes up to 45 kb have subsequently been deleted in EHEC by Red-promoted PCR-mediated island replacement (Campellone and Leong, unpublished).

Table 6

λ Red-promoted PCR-mediated replacement of five targeted EHEC O-islands[a]

Targeted Island	Sequence numbers[b]	Gene(s) putative functions	# of colonies[c] (half the culture)	Growth on kanamycin[d] (# kan[R]/# streaked)	PCR analysis (# verified/ # tested)
O-island #12	353934 - 358187	eaeH putative adhesion	10	8/10	3/3
O-island #77	2710424 - 2712382	Z3025 unknown function	37	10/10	4/6
O-island #103	3315828 - 3317575	Z3664 putative virulence protein	18	18/18	2/3
O-island #130-131	4255687 - 4256420	hopD putative enzyme; degradation Z4695 putative iron storage yheA unknown function	254	18/20	3/3
O-island #169	5311698 - 5313445	Z5815 putative transposase Z5816 putative virulence protein	5	5/5	2/5

a)   O157::H7 genetic elements not present in E. coli K-12

b)   Genbank accession number AE005174 (Perna et al., 2001)

c)   An estimated 0.25 μg of DNA (3.5 μl) was mixed with 50 μl of electrocompetent EHEC cells and shocked as described in Experimental Procedures. Cells were suspended in 3 ml LB following electroporation and grown at 37° for 90 min. Half the culture (1.5 ml) was concentrated by centrifugation and spread on LB plates containing 20 μg/ml kanamycin. In the same experiment, a PCR fragment containing the ΔlacZ::kan allele generated 272 Kan[R] transformants.

d)   Following 24 h growth, candidates were restreaked on LB-kan plates. Among these Kan[R] candidates, recombinant deletion formation was verified by PCR analysis (as described in Figure 1B).

50

Example 5: Drug marker context dependency affects the efficiency of gene replacement

EHEC strains containing the *kan* substitutions shown in Table 6 were
5   further purified by streaking on LB plates containing 40 μg/ml⁻¹ kanamycin.
Interestingly, *kan* substituted islands #12, #77 and #169, while selected on LB plates
containing 20 μg ml⁻¹, did not grow at this higher kanamycin concentration (but did
restreak well at 20 μg ml⁻¹). The other two substitutions (Δisland #103::*kan* and Δislands
#130-131::*kan*), which consistently gave higher frequencies of gene replacement relative
10   to the others, grew well on LB containing 40 μg ml⁻¹ kanamycin. This result suggests
that the position and/or orientation of the drug cassette within the chromosome likely
alters its expression levels. Thus, the low frequency of O-island #169 replacement (see
Table 6) might be due to the influence of neighboring transcripts reading into the *kan*
gene following integration of the Δisland 169::*kan* PCR substrate into the chromosome,
15   or instability of the *kan* transcript due to sequences fused to its 3' end.

To test this hypothesis, deletion of O-island #169 was repeated using a
PCR product that reversed the direction of *kan* transcription within this chromosomal
region (primers 5KO-H-island169L & 3KO-H-island169L in Table 3). In three separate
experiments, (on average) 10-fold higher Kan^R transformants were found when *kan* was
20   reading leftward from the position of O-island #169 (according to the numbering in the
sequence file) instead of rightward. This leftward reading direction of the *kan* gene
places it colinear with other genes in this region (*ytfB* and *Z5814*) and supports the notion
that proper positioning of the drug marker in the chromosome can influence the recovery
of the recombinant.

25   This context effect was also seen with one of the long homology
substrates at the *eae* locus. Initial attempts to generate the EHEC chromosomal
replacement using the fragment from pKM184 (containing *eae::cat*) yielded no
recombinants with repeated attempts. The orientation of the *cat* gene inserted within the
eae flanking regions of pKM184 was determined, and found to be reading opposite to the
30   direction of the endogenous *eae* gene. Thus, a version of pKM184 was constructed
where the *cat* gene was inserted co-directionally with *eae*. Electroporation with this
substrate readily yielded recombinants (see Table 4, lines 1, 4-5).

Thus, the orientation of the drug marker within the target gene site can effect either its expression level (affecting the selection of the recombinant) or its ability to be stably incorporated into the chromosome. With difficult substitutions, both orientations of the drug marker (or the use of properly positioned transcription

5 terminators within the plasmid construct) should be attempted. Context dependent marker expression may be one of the primary causes of the variation seen among Red-promoted gene replacements of the same drug marker placed at different loci along the *E. coli* K-12 and *Salmonella enterica* Serovar typhimurium chromosomes.

10 **Example 6: Extended expression of Red and Gam is mutagenic**

Unlike *E. coli* K-12 and *Salmonella enterica* Serovar Typhimurium, there are no phage transductional protocols for EHEC to place λ Red-generated deletion alleles into clean genetic backgrounds. Thus, it was important to consider the possible mutagenic profile of λ Red expression in EHEC. Somewhat surprisingly, overnight

15 cultures of EHEC containing uncontrolled expression of *red* and *gam* from pKM201 (which does not express *lacI*) showed a 10-fold increase in the rate of spontaneous rifampicin resistance (Figure 4). EHEC containing pKM208 (which expresses the *lacI* repressor as well as the Ptac-*red-gam* operon) showed a significant increase in rifampicin resistance only when incubated in the presence of IPTG overnight.

20 In order to determine the minimum time of Red induction required to generate the hyper-rec phenotype, the frequency of gene replacement was measured as a function of IPTG induction. Figure 5 shows that a 20 minute exposure to IPTG is sufficient to induce the hyper-rec phenotype in EHEC. For most of the experiments reported above, a 1 hour IPTG induction period was used. Thus, EHEC cells were

25 examined to determine whether Red induction for 1 hour induced a mutagenic phenotype. EHEC cells containing pKM208 were exposed to IPTG for 1 hour in a manner identical that used for the preparation of electrocompetent cells, and plated on LB plates containing rifampicin. No increase in Rif^R cells were seen in such preparations when compared to uninduced cultures (see Figure 5, insert). The same was result was

30 seen with EHEC containing pTP223 (data not shown). Thus, while uncontrolled expression of Red and Gam causes a 10-fold increase in mutagenesis, limited expression of Red and Gam required for establishment of the hyper-rec phenotype is not mutagenic.

Example 7: λ Red-promoted PCR-mediated recombination in EPEC

Red and Gam-producing plasmid pKD46 has been reported for use in long homology recombination of plasmids with the EPEC chromosome, but not short homology PCR-mediated recombination. Thus, certain of the above-described plasmids

5   were tested in EPEC for Red-promoted PCR-mediated recombination. It was not possible to transform EPEC with either of the pSC101-origin containing plasmids pKM201 or pKM208. However, it was possible to electroporate EPEC with pTP223. Surprisingly, and unlike the case with EHEC, pTP223 did promote efficient short homology recombination with PCR substrates.

10   This plasmid has been used to construct an EPECΔ*tir* construct (Campellone *et al.*, 2002). A13kb deletion has also been constructed within the locus of enterocyte effacement in EPEC (KC21) in a manner similar to that described above for EHEC (described in Table 1), with the exception that pTP223 was used to supply λ Red and Gam. Also, it has been observed that the heat shock step plays a stimulatory role in

15   Red-promoted PCR-mediated EPEC gene replacement. In one set of experiments, the heat shock step with EPEC resulted in 2-10 fold increase in the number of recombinants (corresponding to a 20-100 fold increase in frequency of recombinants per survivor of electroporation). The effect of the heat shock step is quite variable, however, as the stimulation of recombinants per survivor using the Δ*lac::kan* allele in EHEC to was

20   found to be only 2-4 fold.

Discussion of Examples 1-7

The ability to inactivate or replace a gene of interest in the chromosomes of bacterial pathogens is a critical step in the identification of virulence factors, and in

25   the elucidation of mechanisms of infectivity. However, gene replacement protocols for most pathogenic bacteria prove difficult and time consuming. The above Examples demonstrate that λ Red can be utilized for the manipulation of the chromosomes of EHEC and EPEC. The value of such a system has been demonstrated by observing a difference in phenotype for bacteria expressing an engineered virulence factor from a

30   plasmid versus its normal chromosomal location.

Two schemes have been presented for engineering the chromosomes of EHEC and EPEC. In one case, plasmids are constructed that contain a drug marker

flanked by upstream and downstream regions of the target gene. The plasmid is digested with the appropriate restriction enzymes, liberating the recombinant fragment (substrate) which is electroporated into Red-producing cells. The frequency of simple plasmid transformants can be decreased by digesting the plasmid with backbone-specific

5   restriction enzymes prior to electroporation. As an alternative, the marked deletions can be constructed in plasmids with conditional replicons, such as those that require the trans-acting π protein (the *pir* gene product) for replication (Metcalf *et al.*, 1996), though such plasmids were not used in this study. Electroporation of a linear DNA containing the marked deletion generates a gene replacement without the need for plasmid co-

10   integrant formation and resolution.

In the case where a counter-selectable marker is placed on the EHEC or EPEC chromosome (*e.g.*, *sacB*), the target gene can be replaced with any site-directed mutant generated *in vitro* simply by electroporation of a linear fragment containing the mutated allele. While this scheme requires prior construction of the deleted or modified

15   allele on a plasmid, it is useful in situations where multiple mutant alleles need to be crossed on to the chromosome, as was presented here with the hybrid *tir* constructs. In addition, precise in-frame deletions can be easily constructed (see Table 5). The benefits of generating precise deletions for genetic analysis are clear relative to transposon mutagenesis procedures, where one cannot be if sure the entire gene is

20   inactivated, or whether an insertion affects downstream functions within an operon (*i.e.*, polarity effects). Finally, the Red-producing plasmids described here, (including pTP223 which does not have a temperature sensitive origin of replication) are unstable following induction with IPTG (10-50% of the transformants lose the plasmid). This is surprising benefit, as gene replacement and plasmid curing occurs simultaneously after

25   electroporation, and such transformants can be found by screening. Transformants cured of the plasmid and modified at the gene of interest are readily available for *in vitro* and *in vivo* analyses.

The expression of native and mutant genes from low copy number plasmids (in the context of a chromosomal deletion of the gene) has been a common

30   method to assess the function of virulence factors in bacterial pathogens. One must be careful to note, however, that expression from low copy number plasmids does not always reflect the phenotype of native (chromosomal) expression patterns of these genes. The expression of hybrid Tir molecules reported here is a prime example.

Despite the similarities between chromosome and plasmid-encoded expression of these modified genes, the Tir-HHH$_{NBS}$ hybrid protein exhibited its mutant phenotype (extended pedestals) only when it was expressed from the chromosome.

5　　　It is currently unknown whether the differences within this Tir hybrid that create the long pedestal phenotype are directly related to the interaction of the cytoplasmic domains with host cell components, due to changes in Tir structure in the context of the chimera, or from altering the association of other substrates with the type III apparatus. Clearly, the masking of the long pedestal phenotype associated with overexpression of Tir-HHH$_{NBS}$ from a plasmid-encoding locus highlights the

10　importance of directing the expression of bacterial products from their endogenous loci. The ability to engineer the chromosomes of pathogenic *E. coli* with lambda Red recombination greatly facilitates such genetic analyses.

In the second scheme for EHEC or EPEC gene replacement, one-step gene or pathogenicity island deletion is performed by electroporation of PCR fragments

15　containing a drug marker flanked by 40 bp of target DNA. The ease of this system makes it preferable when multiple target genes must be precisely deleted for genetic or *in vivo* analysis. The versatility of the system is highlighted by the range of chromosomal segments that λ Red can act on, deleting pathogenicity islands as small as 733 bp and as large as 45 kb. However, in performing short homology recombination in

20　EHEC, a reduction in efficiency relative to that seen in *E. coli* K-12 was noticed. One possibility to explain these results is that the λ Gam protein might not be as active on EHEC RecBCD as it is for *E. coli* K-12 RecBCD. This seemed unlikely given the conservation of the *recBCD* genes between these two species (97-98% conservation). Nonetheless, a EHEC Δ*recC::cat* knockout was made and tested its ability to perform

25　Red-mediated SHR. Deactivating RecBCD function by deletion of *recC* did not stimulate λ Red recombination in EHEC at the *lacZ* locus (data not shown). Thus, to a first approximation, λ Gam works as efficiently with EHEC RecBCD as it does with *E. coli* K-12 RecBCD. Another possibility is that the *red* functions from the endogenous lambdoid EHEC prophage 933W would be better suited than wild type *λ red* for gene

30　replacement in EHEC. This seems unlikely given the high degree of conservation between λ and 933W *red* genes (99.6% identity), and was not tested.

Despite this lower frequency of Red recombination in EHEC (as judged by recombination in the *lacZ* locus), λ Red works at an efficiency that appears to allow any non-essential regions of the chromosome to be manipulated. This is true for EPEC as well when *red* and *gam* are expressed from plasmid pTP223. It is curious that

5   pTP223 works well in EPEC, but not in EHEC or *E. coli* K-12 for PCR-mediated Red recombination. Perhaps in EPEC, pTP223 does not replicate by extensive rolling-circle replication as was seen for *E. coli* K-12, thus preventing linear multimers of the plasmid from interfering with Red function. This hypothesis, however, has not been tested.

Another interesting observation reported here is the mutagenic phenotype

10   of constitutive *red* overexpression. Perhaps the annealing function of Bet interferes with mismatch repair pathway of *E. coli* by reannealing the unwound ssDNA generated by UvrD, thus interfering with the progression of the mismatch repair process (see Hsieh (2001) for review). Even though Red can substitute for RecBCD in recombinational repair and conjugation (Murphy, 1998), the mutagenesis associated with Red may

15   explain why many bacterial species do not possess a constitutive phage-like recombination system as their primary recombination pathway, and instead employ the more rigorous Chi-activated RecBCD dsDNA exonuclease to generate recombinant intermediates (a pathway that does not involve a known Bet-like ssDNA annealing protein).

20   The mutagenic phenotype of constitutive Red expression (and possibly RecET as well) highlights the importance of controlling these functions *in vivo* when using them for gene replacement in pathogenic bacteria (*i.e.*, limiting their expression to a recombinogenic window). EHEC and EPEC containing Red and Gam-producing plasmids may also be amenable to ssDNA oligo-directed chromosomal alterations and *in*

25   *vivo* cloning by gap repair, as has been demonstrated with *E. coli* K-12 strains expressing phage recombination functions (Ellis *et al.*, 2001; Lee *et al.*, 2001; Zhang *et al.*, 2000). The λ Red recombination system can be adapted for use in *Pseudomonas aeruginosa*, and other bacterial pathogens. Manipulation of the chromosomes of other pathogenic organisms allows analysis of a variety of bacterial pathogenic mechanisms.

30   Moreover, the genomes of several clinically relevant bacteria have been sequenced, and thousands of new genes are now available for genetic analysis.

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30 <u>Equivalents</u>   Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.